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THE EFFECTS OF HYDROSTATIC PRESSURE ON LUMINESCENT EXTRACTS OF THE OSTRACOD CRUSTACEAN, CYPRIDINA

J. RAMSEY BRONK, E. NEWTON HARVEY
AND F. H. JOHNSON
The Biological Laboratories, Princeton University

SEVEN FIGURES

The effect of hydrostatic pressure on an isolated luminescent system is of special interest because moderate pressures, around 5000 lbs/in^2, are known to affect many biochemical reactions in vitro as in vivo. Moreover Brown, Johnson and Marsland ('42) have demonstrated definite effects of pressure on the luminescence of luminous bacteria, depending on the temperature of the organisms. In general, pressure and temperature relations are so intimately connected that no pressure study is complete without a thorough knowledge of the effect of temperature on the system (see Johnson, Eyring and Polissar, '53). With the partial analysis of the temperature relations of Cypridina luminescence already available (Chase and Lorenz, '45), two of us (E.N.H. and F.H.J.) started pressure studies in 1950, but the problem turned out to be more complicated than expected. The conditions under which pressure affects the purified Cypridina luminescent system have now (1952) been studied further and form the basis of this paper. Among other things, the technical problem of satisfactorily mixing substrate and enzyme solutions while under pressure has now been solved by a simple device, as described presently.

Chemical studies have indicated that the chemiluminescent system of the ostracod crustacean, Cypridina hilgendorfii, is a relatively simple one, involving only oxygen, luciferin and

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luciferase. Unlike the fire-fly, no conditions have been found where adenosinetriphosphate and certain cations are necessary for luminescence in Cypridina extracts, although more light is emitted in solutions containing chloride and some other ions than in their absence.

Both luciferase and luciferin can be isolated from dried Cypridinas and partially purified. The luciferin has been found to oxidize in two ways, spontaneously in aerated water without light production, and with luminescence if luciferase is also present. The spontaneous oxidation results in an oxidation product which can be reduced by the addition of hydrogen, provided the oxidized luciferin solution has not stood too long. The oxidation in presence of luciferase gives rise to products irreversible by hydrogenation methods (Anderson, '36).

Both types of oxidation ordinarily occur simultaneously, and both proceed as first order reactions. By measuring the decrease in light intensity I with time in a mixture of luciferin and luciferase, decay curves can be obtained which are straight lines when log I is plotted against time. The slope of the line is the combined velocity (or rate) constant, and under standard conditions, is proportional to the concentration of active luciferase. The area under the decay curve represents the total light emitted and, again under standard conditions, is a measure of luciferin concentration.

Thus, Cypridina luminescence results from relatively simple chemical reactions whose kinetics can be investigated by measuring light intensity alone and the effects of pH, salts, inhibitors, temperature, etc. on the kinetics of the purified luciferin-luciferase reaction have been extensively studied by Anderson, Chase and others (see Harvey, '52). It is this well known material which has been used in the present study.

METHODS AND MATERIAL

For light measurements a RCA-1P21 photomultiplier tube with negligible dark current was used. A suitable power supply, stabilized with individual gas discharge tubes and a

voltage regulator on the A.C. line, maintained 100 volt potential difference between dynodes. The photoelectric current was read on a large milliammeter and found to be proportional to light intensity at 5 different sensitivities. It is important that the photomultiplier tube does not receive too much light and that the whole light sensitive surface be illuminated in order to obtain consistent results. These conditions were attained by the use of neutral filters between the light emitting solution and the photomultiplier tube.

Whether the experiment took place at one atmosphere or under increased pressure the Cypridina luciferin and luciferase solutions were always contained in a steel pressure chamber with a quartz window through which the light reached the photomultiplier tube. For every measurement the pressure chamber was placed in a definite position on a shelf in a glass water bath at a fixed distance from the phototube, which was itself immovable. Thus no light intensity changes could be due to change in relative positions of luminous solution and light sensitive surface of the tube. In the great majority of cases temperature was maintained constant to $\pm$ 0.5°C.

Pressures of 2500 to 7500 lbs./in^2 were applied with a hydraulic pump, transmitted by a copper tube filled with oil, as is customary in pressure studies (see Johnson, Flagler, Simpson and McGeer, '51).

In the earlier experiments the pressure chamber, which held about 18 ml, was quickly filled with luciferin solution mixed with a small amount of luciferase, and the measurements begun as soon as the chamber could be placed in position before the phototube, an operation which took about one minute. The pressure chamber and the solutions had previously been equilibrated at the proper temperature before filling the chamber. With low luciferase concentration a long lasting light was obtained and pressure could be applied and released many times during an experiment, as shown in figure 2.

In the later experiments the mixing of luciferase was effected after the chamber had been closed and connected to the hydraulic pump. For mixing under pressure an aluminum tube just large enough to fit the pressure chamber was used. It was closed at one end and machined inside so as to make a shelf about one quarter its length from the open end, as shown

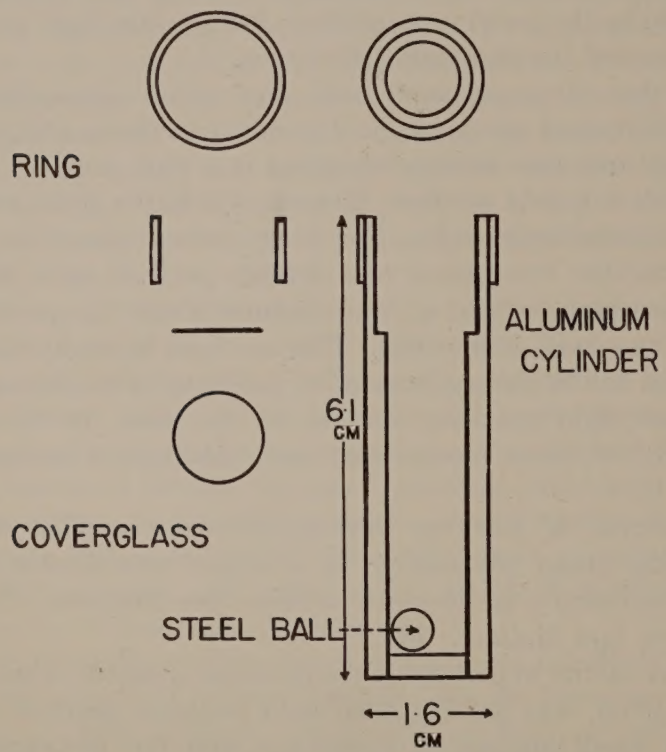

Fig. 1 Details of the metal tube for mixing luciferin and luciferase solutions under high hydrostatic pressures. Explanation in text.

in figure 1. The shelf was vaselined and the tube then filled to the shelf with luciferin solution. A one-half inch round glass cover slip could then be laid on the shelf, and pressed down, holding the luciferin in the lower chamber. After removing excess luciferin, luciferase solution was placed in the upper two-fifths volume and the tube closed with two thick-

nesses of cellophane held in position by an aluminum ring. The tube, holding about 5 ml, was then placed in the pressure chamber with the cellophane end next to the quartz window. If a steel ball had been previously placed in the larger compartment of the tube, mixing of the luciferin and luciferase could be easily accomplished by shaking the pressure chamber, whereupon the ball displaced the cover slip. After mixing, the chamber was placed in position in the water bath and the measurements begun about 15 seconds from the start of

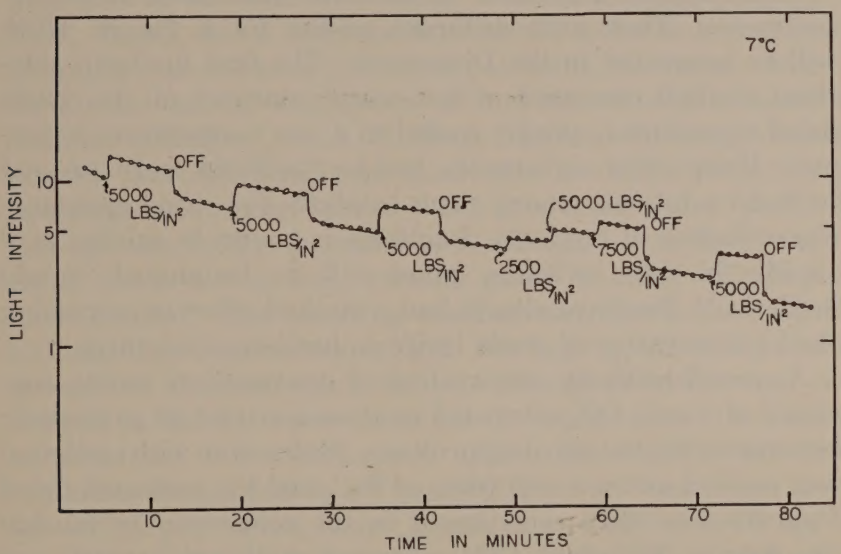

Fig. 2 Effect of various pressures on crude luciferin + "precipitated" luciferase mixture at 7°C. Log light intensity vs. time.

mixing. This simple device worked perfectly, provided care was taken to shake violently enough to effect complete mixing of the two solutions.

The luciferase solutions were prepared in two different ways. That used by E.N.H. and F.H.J. in the 1950 experiments was an extract of the whole dried Cypridinas, dialyzed against distilled water for many weeks, as described by Chase, Schryver and Stern ('48). The luciferase used in the 1952 experiments was prepared by the method of McElroy and Chase ('51),

carried only through the acetone and ammonium sulphate precipitations. The precipitate was dissolved in a small amount of water and frozen for preservation. A stock solution for the experiments was made by mixing 1 ml of the frozen material (after melting) with 100 ml distilled water. The diluted luciferase was kept in a refrigerator until dilutions with 0.15 M phosphate buffer at a pH of 7.2 were made for use in an experiment. Unless otherwise stated "precipitated" luciferase was used in the experiments reported in this paper.

Three different purities of luciferin were used in the experiments. They gave different results for a reason which will be suggested in the Discussion. The first luciferin solutions studied consisted of hot water extracts of the whole dried Cypridinas, quickly cooled to a low temperature before use. Many other substances besides luciferin were present in these solutions, among them catalysts for rapid spontaneous oxidation of luciferin. Luciferin is known to oxidize very rapidly in these extracts, which will be designated "crude luciferin." Pressure always had a marked effect in increasing the light intensity of crude luciferin-luciferase mixtures.

A second luciferin preparation of intermediate purity consisted of a cold CO_2 saturated methanol extract of previously benzene-extracted dried Cypridinas. Extraction with methanol was carried out in a cold room at 0°C. and the methanol-dried Cypridina mixture centrifuged in the cold room to remove the debris. The clear CO_2 saturated methanol extract was kept free of molecular oxygen in a special vessel under CO_2 so that small amounts could be removed when needed. Actually, 10 cm^3 samples were removed, evaporated to dryness by a water aspirator, and the residue dissolved in 0.15 M phosphate buffer saturated with CO_2 (resultant pH = 6.5) and kept in another special vessel under CO_2. Small amounts of the aqueous extract were then used in the pressure chamber when needed. When 3 ml of this luciferin was mixed with 2 ml of the luciferase solution at pH 7.2, the resultant pH was 6.8. Pressure had little effect on the luminescence of the methanol extracts of dried Cypridina, which will be referred

to as "methanol luciferin," considerably purer than the crude water extract. Certain anomalies in the kinetics of luminescence with these extracts, however, indicated that they contained substances which act as inhibitors (Johnson and Eyring, to be published); i.e., the velocity constant of the reaction increased with dilution of the luciferin solution, as explained below.

The third type of luciferin solution used was purified by Anderson's ('35) method, with two cycles of benzoylation. Two sets of material were used. The first, supplied by Dr. H. S. Mason, was a butyl alcohol extract and was kept under hydrogen purified over platinized platinum. As it was needed, the alcohol extract was drawn off and diluted with a suitable quantity of buffer. Dr. A. M. Chase supplied the second batch of pure material which was used with the improved mixing technique mentioned above. His material — also purified through two cycles by Anderson's method — was first evaporated down with a high vacuum pump to draw off all the butyl alcohol. The resulting residue was dissolved in 0.1 N HCl and kept in an ice water bath. For a group of runs a small amount of the pure material was diluted in buffer and remained in the ice water bath for the duration of the series of runs (not over two hours). Chase has found ('48) that the luciferin solution in HCl at 0°C. does not lose more than 1% or 2% activity an hour. In buffer at a pH near 7.2, however, it is oxidized much more rapidly. The pH of all luciferin-luciferase mixtures was determined at the end of a run, using the Beckman pH meter.

With the above luciferin, which will be referred to as "purified luciferin," pressure has an effect on light intensity only under special conditions.

RESULTS

Any substance or factor may affect luciferin-luciferase luminescence by changing the light intensity or the velocity constant or the total light emitted. Total light has not been measured in this study but changes in light intensity and

rate constants have been recorded for all three purities of luciferin solution. It has already been intimated that the results of pressure experiments depend on the purity of the luciferin and the luciferase.

Light intensity. Increased hydrostatic pressure increases the light intensity of crude extracts of luciferin and luciferase at all temperatures studied (14° to 34°) but has no effect on the purified luciferin-luciferase reaction, except under special conditions.[1] The contrast is particularly striking from inspection of figures 3 and 4. It will be noted that the pressure effect is reversible (see figs. 2, 3, 6 and 7).

An analogous difference in the effect of cyanide on the crude and purified luciferin-luciferase luminescence has been previously observed. For example, Harvey ('17) found no effect of cyanide (even in 0.2 M concentration) on crude luminescent mixtures, whereas Giese and Chase ('40) observed that cyanide, in very dilute solution, combines irreversibly with pure luciferin, thus decreasing the total luminescence.

Even the kinetics of crude and purified luciferin is different. Amberson ('21) observed that when crude luciferin and luciferase were studied the rate constant varied not only with luciferase concentration as is to be expected, but also with luciferin concentration if the dilution of the crude luciferin was made with water, an effect contrary to kinetic theory. If the dilution of the crude luciferin was made with oxidized luciferin, i.e., an oxidized extract of the dried Cypridinas, then the rate constant was the same with different luciferin concentrations. This observation has been confirmed in the present study with crude luciferin and also observed with the methanol luciferin. It is understandable on this

[1] In the earlier experiments (by E. N. H. and F. H. J.), using luciferin purified by one cycle of benzoylation, with dialyzed luciferase, a marked reversible decrease in light intensity under pressure was noted at 10–11°, a slight decrease at 27°, a slight increase at 37–38° and a marked permanent increase at 43–44°C. There were three differences between the solutions used in these experiments and in the later ones: (1) the luciferin was a different sample; (2) the luciferase was prepared by dialysis; (3) the buffer solution contained 1% NaCl. It was determined by experiment that the 1% NaCl was not responsible for the result, but no further analysis has been attempted.

basis (Johnson and Eyring, to be published) that the crude and methanol extracted luciferin solutions contain an inhibitor (or inhibitors) whose action is reversible on dilution. No such effects of dilution are observed with purified luciferin (Chase and Harvey, '42).

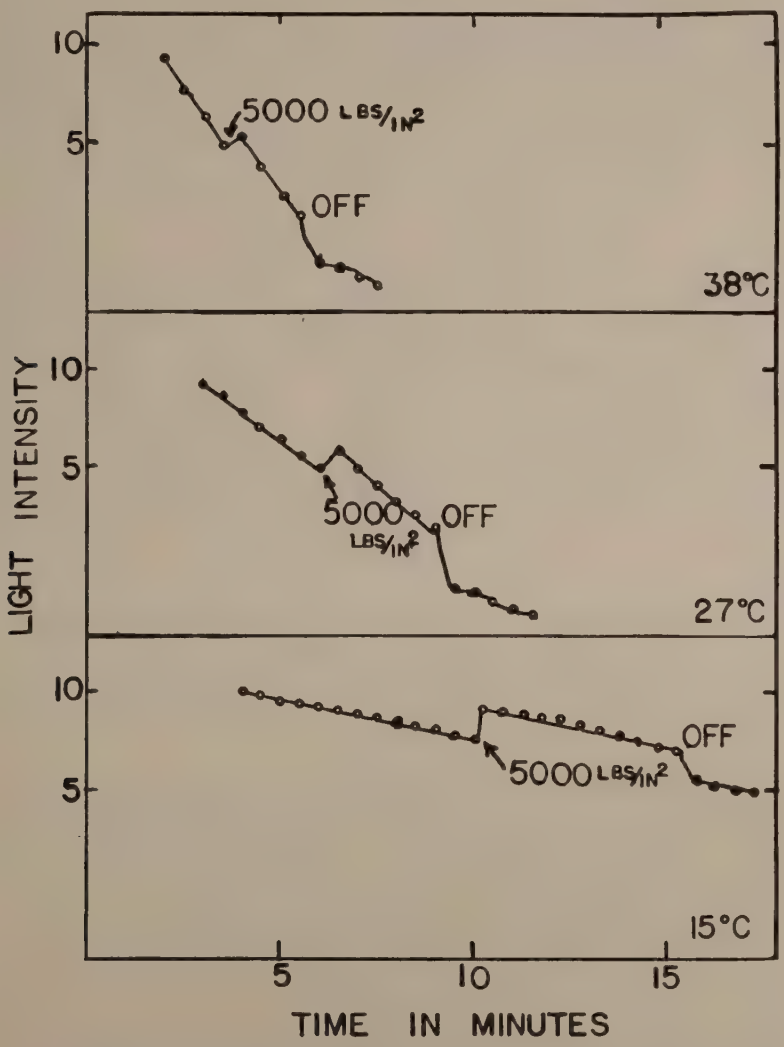

Fig. 3 Effect of pressure on crude luciferin + "precipitated" luciferase mixtures at three different temperatures. Log light intensity vs. time.

The positive pressure effects on the luminescence intensity of crude luciferin and luciferase are not exhibited in the methanol luciferin experiments, as shown in figure 5, although the latter do exhibit the anomalous variation of rate constant when luciferin is diluted with water.

The effect of pressure on light intensity in crude luciferin solutions might be attributed, in part, to conversion of some of the spontaneously oxidized luciferin to luciferin, which,

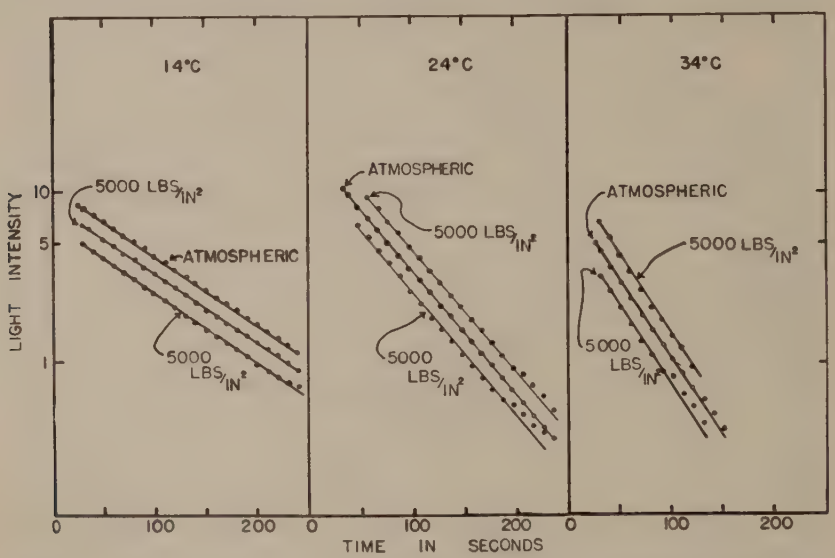

Fig. 4 Effect of pressure on purified luciferin + "precipitated" luciferase mixtures at three different temperatures. Log light intensity vs. time.

in presence of luciferase and under pressure, then oxidizes with additional light production. If pressure speeds the conversion of oxidized luciferin to luciferin more than it does the opposite reaction, then an effect of pressure on light intensity should be demonstrable with purified luciferin, provided the latter has stood long enough for a considerable amount of spontaneously oxidized luciferin to accumulate. A case in point is shown in figure 6, where the luciferin was mixed outside the pressure chamber, then placed in the chamber at 34° and pressure applied late in the course of the

reaction when the log I vs. time plot no longer gave a straight line. Similar experiments at three additional temperatures, 44°, 24° and 14°, were also made. Purified luciferin which had stood for a long time in order that reversibly oxidized luciferin might accumulate was used in the experiments. All three curves show reversibly increased light intensity with pressure, most marked at the higher temperatures. In general many experiments indicate that the positive pressure effect

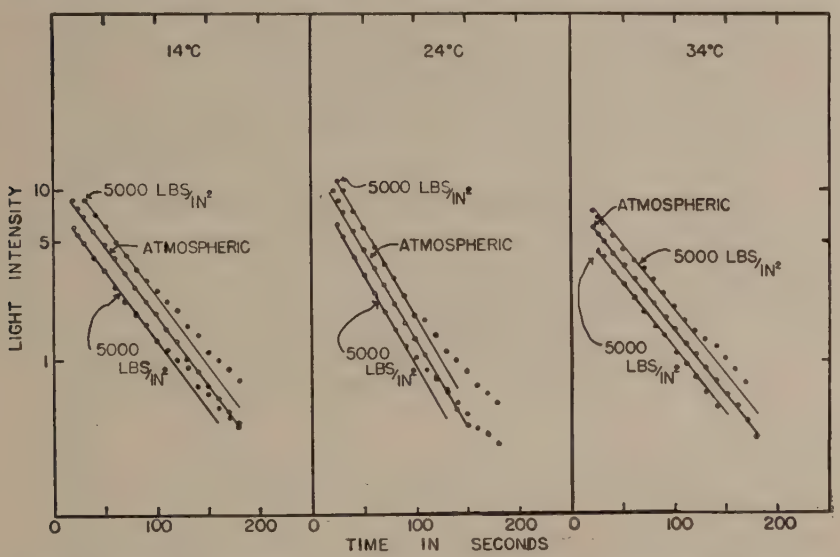

Fig. 5 Effect of pressure on methanol luciferin + "precipitated" luciferase at three different temperatures. Log light intensity vs. time. The luciferase concentration was less in the experiment at 34°.

is more pronounced the higher the temperature and the higher the pH, both conditions which favor accumulation of reversibly oxidized luciferin.

Apart from the spontaneous oxidation of luciferin in more alkaline solutions, it was not observed in these experiments that pressure acted differently at pH values between 6.5 and 7.6.

One other interesting effect of pressure on light intensity was observed with crude luciferin solutions. When pressure was applied, the light intensity would sometimes "over-

shoot,'' i.e., become considerably increased for a few seconds and then decrease to the proper value under pressure. Similarly the decrease in light intensity when pressure was removed would ''undershoot'' for a few seconds and then return to the proper value at atmospheric pressure. The ''under-

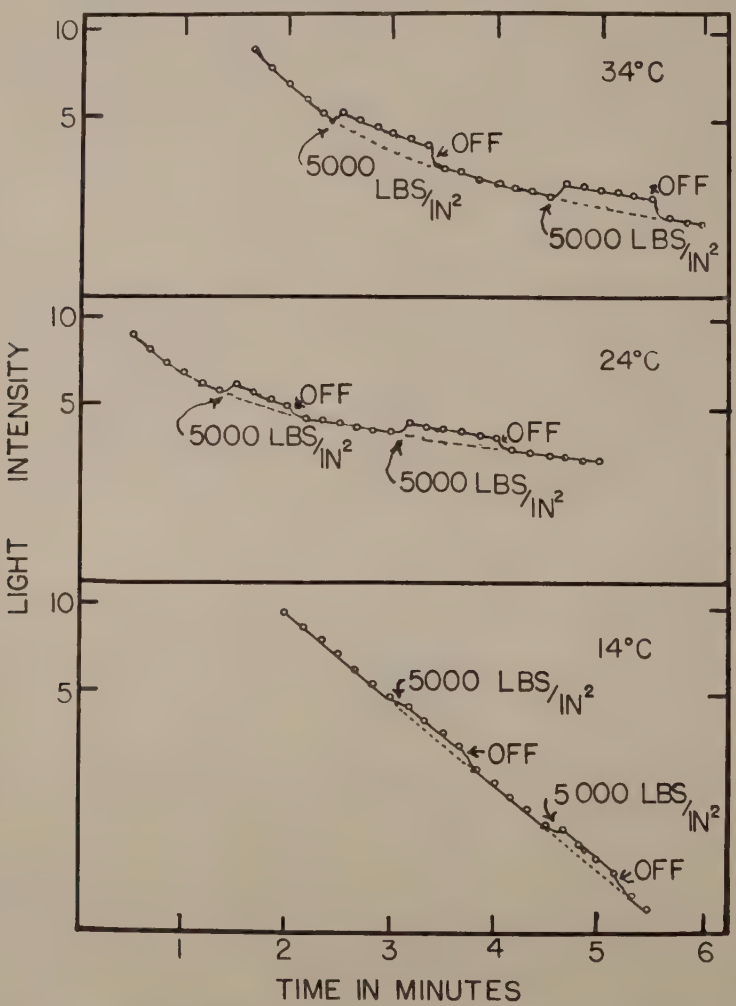

Fig. 6 Effect of pressure on purified luciferin solution allowed to stand until considerable oxidized luciferin had accumulated, and then mixed with ''precipitated'' luciferase. Log light intensity vs. time.

shooting'' was noticed by Brown, Johnson and Marsland ('42) with luminous bacteria after subjection to hydrostatic pressures and called the ''blackout.''

Velocity constant. It will be noted from figures 4 and 5 that the velocity constant does not change under pressure when purified luciferin and methanol luciferin are studied. The precise change in slope under pressure when crude luciferin is used (figs. 2 and 3) is difficult to establish since the log plot soon changes from a straight line and becomes curved. The departure from a straight line is observed under conditions where a very considerable amount of reversibly oxidized luciferin has accumulated and is being converted to luciferin by reducing substances. The luciferin is then reoxidized with light emission. This process results in a long continued weak luminescence in solutions whose light might be expected to disappear according to the exponential decay law.[2] Deviation from a straight line toward the end of this reaction may be observed in the methanol luciferin experiments at higher temperatures, illustrated in figure 4.

In experiments with crude luciferin solutions which do exhibit straight line decay curves, no change in slope could be detected under pressure, as illustrated in figure 7, although the reversible increase in light intensity upon application of pressure was marked. As judged by the observed velocity constant there appears then to be no effect of pressure at any temperature on the activity of luciferase, prepared by the precipitation method of McElroy and Chase ('51).

In the earlier experiments (by E.N.H. and F.H.J.), using dialyzed luciferase with crude luciferin, some of the results

[2] This terminal weak emission of light might be interpreted as the inhibition of luciferase activity by the reaction product, spontaneously oxidized luciferin, but in the kinetic studies of Chase and Harvey ('40) no evidence of such an effect was observed when spontaneously oxidized luciferin was added to luciferin-luciferase mixtures. If pressure acted to reverse the inhibition of luciferase by the reaction products, the rate constant should always increase under pressure, but this is not the case.

were different. Thus, on some occasions a decrease in velocity constant under pressure was noted at low temperatures (10°) and an increase in velocity constant under pressure was noted at a high temperature (45°).

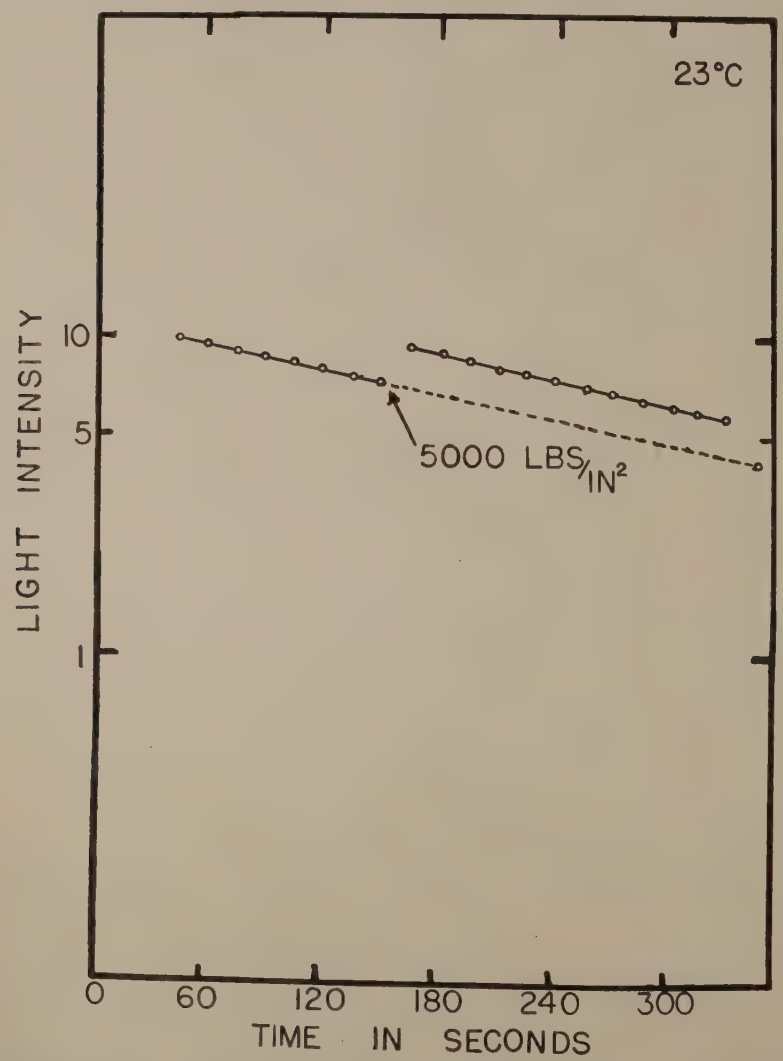

Fig. 7 Effect of pressure on crude luciferin and "precipitated" luciferase mixture which exhibits logarithmic decay, showing no change in slope under pressure. Log light intensity vs. time.

DISCUSSION

The pressure effects on Cypridina luminescence are of most interest for comparison with the pressure and temperature relations of luminous bacteria, where the presence of luciferin and luciferase is assumed, although they have not been isolated or demonstrated. With luminous bacteria, the effect of temperature is to increase the light intensity to a maximum, the optimum temperature, and to decrease reversibly the light intensity above the optimum, provided the organisms have not been kept too long at the high temperature. The optimum depends on the species of bacterium and the chemical environment. Brown, Johnson and Marsland ('42) have found that below the optimum temperature for bacterial luminescence, hydrostatic pressure decreases and above the optimum it increases the light intensity. At the optimum, pressure has no observed effect.

These pressure-temperature relations were explained as follows. Within the bacterium there is a continuous formation and oxidation of luciferin, represented by the equation:

$$\text{precursors} \rightarrow \text{luciferin} + \text{luciferase} \rightarrow \text{products} + \text{light} \qquad (1)$$

A steady state must be attained for each temperature, since the intensity is constant. Below the optimum temperature, increasing temperature increases but high pressures slow the overall rate. Hence pressure decreases the light intensity at every temperature below the optimum. Above the optimum the lowered steady state luminescence intensity is due to reversible heat denaturation of the luciferase, thus reducing the light intensity as indicated by the equation:

$$\text{native luciferase (An)} \rightleftharpoons \text{denatured luciferase (Ad)} \qquad (2)$$

Pressure applied at temperatures above the optimum shifts the An $\rightleftharpoons$ Ad equilibrium to the left, with increase in the amount of active luciferase and hence increase in light intensity. The opposite effects of temperature on the two reactions just balance at the optimum temperature and pressure has no effect at this point. Since pressure slows reaction (1) and shifts the equilibrium of (2) toward An, it is obvious

that reaction (1) proceeds with volume increase and that denaturation (Ad formation) also occurs with volume increase. The volume increase (cm^3/mol) has been calculated from the data by Eyring and Magee ('42).

For comparison with Cypridina luminescence it is important to know the optimum temperature for activity of luciferase. In the above studies on the kinetics of Cypridina luciferin oxidation the constant k actually measured for the decay of luminescence is an apparent or composite constant equal to k_1, the rate constant for the oxidation of luciferin in presence of luciferase, plus k_2, the rate constant for spontaneous oxidation of luciferin. Temperature may be expected to change the rate constants for each of these reactions, thus influencing the rate of the light emitting process. The efficiency of the light emitting process, i.e., the fraction of the molecules reacting which actually emit a photon, may also be affected by temperature and would affect the total light emitted.

Quantitative data on these temperature effects for Cypridina luminescence have been obtained by Chase and Lorenz ('45), who analyzed the data as two simultaneous first order reactions with velocity constants, k_1 and k_2, without including possible quenching effects.

Under the experimental conditions of Chase and Lorenz the composite oxidation velocity constant (k) showed an optimum at 27° and the calculated velocity constant for the luminescent oxidation k_1 an optimum at 23°. However, it should be borne in mind that if different concentrations of a catalyst for the spontaneous oxidation of luciferin were present in our experiments, not only would k_2 be different from that found by Chase and Lorenz (at any one temperature), but the optimum for k (observed) would also differ. However, k_1 should not be affected by this factor alone, nor should the optimum temperature for luciferase activity indicated by k_1 change, when analyzed in the above manner.

It will be observed from figure 4 that the slope of the decay curve and hence the composite rate constant k is greater at 34° than at 24°, whereas Chase and Lorenz found the k at

34° to be less than at 24°. As indicated above, the greater slope at 34° could mean either a more rapid spontaneous oxidation of luciferin in the present experiments, or a greater heat stability of the enzyme when prepared according to the procedure used in this work. Quantitative data on the total light emitted at the different temperatures are needed to resolve these possibilities. Such data are needed, also, before any complete interpretation of the described effects of pressure can be advanced. The possibility remains that the failure of increased pressure to cause observable changes in the velocity constants is due to compensatory volume changes in the various reactions that may influence both the rate and efficiency of the overall process, although this seems unlikely.

The pressure studies of Werbin and McLaren ('51) on isolated enzyme systems, including chymotrypsin acting on casein, and trypsin acting on beta-lactoglobulin, indicate exactly opposite effects, an increase in rate constant with pressure for the former and a decrease for the latter. Indeed, these authors found that pressure affected the same enzyme (trypsin) differently, depending on the substrate. There is thus no reason to expect the isolated luciferin-luciferase system of Cypridina necessarily to behave like the bacterial luciferin-luciferase system within the organism.

SUMMARY

The effect of hydrostatic pressures of around 5000 lbs./in.^{2} on the luminescence of mixtures of Cypridina luciferin and luciferase depends on the purity of the luciferin and the mode of preparation of the luciferase. With highly purified luciferin, and luciferase prepared by precipitation, pressure does not affect the observed rate constant or the luminescence reaction during oxidation of luciferin until a considerable amount of spontaneously oxidized luciferin has accumulated. Then pressure reversibily increases the light intensity at all temperatures tested (14° to 44°).

The positive pressure effect is understandable by shift of the equilibrium to the left for the non-luminous transforma-

tion: luciferin $\rightleftharpoons$ oxidized luciferin, thus increasing the concentration of luciferin. Oxidation of luciferin with luciferase at the higher luciferin level results in greater light emission. The direction of the shift in equilibrium under pressure would indicate that the formation of spontaneously oxidized luciferin (during non-luminous oxidation) occurs with increase of volume.

With crude luciferin and luciferase preparations, and especially at the higher temperatures, in which spontaneous oxidation of luciferin is rapid, the effect of pressure is to reversibly increase light intensity as soon as the reaction can be measured. In certain experiments the rate constant increases under pressure at high temperatures and decreases at low temperatures.

Experiments with partially purified luciferin, and luciferase prepared by dialysis, have revealed conditions in which pressure decreases the light intensity at low temperatures and increases the light intensity at high temperatures.

The relation of these findings to pressure effects on other pure enzyme systems and to previous studies on pressure and light intensity of luminous bacteria is discussed. It must be concluded that with different substrates for the same enzyme, or if inhibitors are present, or if many rate constants and equilibria are involved in the measured results, the prediction of the effects of pressure on biological systems is precarious.

ACKNOWLEDGMENTS

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TEMPERATURE-PRESSURE STUDIES ON THE CARDIAC RATE IN TISSUE CULTURE EXPLANTS FROM THE HEART OF THE TADPOLE (RANA PIPIENS)

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THREE FIGURES

Especially since 1934, high pressure has provided a very useful method for analyzing physiological processes in various cells and tissues (cf. Marsland, '42); and studies in which both temperature and pressure have served as experimental variables have been even more revealing (cf. Brown, '34; Brown, Johnson and Marsland, '42; Marsland, '50). However, these techniques have not previously been applied to tissue culture preparations.

Explants of various tissues from young frog tadpoles were tested as to viability and growth under tissue culture conditions and cardiac tissue was finally chosen for the experiments. This tissue proved to be exceptionally hardy under the experimental conditions (see later). Also, the steady rhythmic beating of the heart fragments, which continued more or less indefinitely in the cultures, provided an opportunity for studying the cardiac rate at temperatures between 5° to 40°C., and at pressures ranging up to 16,000 lbs./in.^{2} (Landau and Marsland, '50). And finally, previous studies dealing with pressure effects on rate processes — particularly the work of Edwards and Cattell ('28) on the cardiac rate of the intact heart (frog and turtle), and that of Pease and

[1] This work was supported by Grant no. C807(c), from the Cancer Research Branch of the National Institute of Health, United States Public Health Service.

Kitching ('39) on the ciliary rate in Mytilus — have provided an interesting background for the present study.

MATERIALS AND METHODS

Tissue culture techniques [2]

The heart tissue of R. pipiens tadpoles 15–22 mm in length was used in all experiments, and all the operations were performed under sterile conditions. The tadpoles were first bathed in a supersaturated solution of sulfathiazol in distilled water for a period of 15–20 minutes and then transferred individually into the first of two ovular depression slides containing approximately 5 cm^3 of Standard Holtfreter Solution, to which penicillin (1000 units per cubic centimeter) had been added. In the first dish the tail of each animal was cut off to reduce wriggling during subsequent operation of removing the heart tissue, which operation was performed after transferring the animal to the second dish.

In a few cases the whole heart including the sinus venosus and bulbo-conus arteriosus was excised and cultured. With larger hearts, however, the ventricle was split longitudinally, thus forming two fragments, one containing the conus and the other the sinus. In either case a regular beating developed after the culture was established; and no consistent differences between the preparations were observed.

After excision, the tissue was placed immediately in another bath, identical with the former two, and thence the explant was mounted in medium on a three-fourths inch no. 1 round cover-slip which was sealed and stored, using the standard double cover-slip technique.

Culture media

For most of the experiments the heart fragments were cultured in a gelatin-containing culture medium, which was prepared as follows. One drop of a 1:3 mixture of a 9-day chick

[2] Cordial thanks are extended to Miss Gladys Cameron, who advised us so effectively in regard to the tissue culture procedures.

embryo extract (made with Standard Holtfreter Solution containing 1000 units of penicillin per cubic centimeter) and 4% gelatin (Difco) in distilled water was added to an equal drop of standard chicken plasma. However, in a few of the early experiments frog blood serum was substituted for the gelatin solution. The gelatin medium was easier to prepare and was more stable during sterilization (in the autoclave at 15 lbs. pressure for 30 minutes). The outgrowth of fibroblasts in the gelatin medium, although not as abundant as in the serum medium, indicated that the cultures were in good condition. Also in the gelatin medium, there was less tendency for the fibroblast outgrowth to obscure the cardiac tissue.

All cultures were kept in a refrigerator at 9°–12°C. for at least 24 hours before use in the pressure chamber.

Mounting the cultures in the microscope-pressure chamber

The microscope-pressure bomb was similar to the one described by Marsland and Brown ('36). In the present work, however, the explant was mounted in a small cylindrical lucite vessel, which then was placed inside the bomb. The lucite chamber, which was open at one end, had a height of one-half inch, and an outside diameter of three-fourths inch, which corresponds to the diameter of the cover-slips used in preparing the cultures. In mounting a culture, the rim of the open end of the lucite vessel was painted with melted stopcock grease, the chamber was filled with Holtfreter Solution, and the coverslip bearing the explant was inverted and pressed into place over the open end of the lucite vessel. Now the vessel containing the tissue bathed in Holtfreter Solution was placed, cover-slip side down, in the pressure bomb. Only the thickness of the cover-slip intervened between the tissue and the lower window of the bomb and thus the tissue could be brought into the focus range of the objective of an inverted microscope.

Completely sterile precautions proved unnecessary in transferring the cover-slip mounts to the lucite chambers, since

no contamination difficulty was encountered once the culture preparation stages were passed. Control preparations using sterilized lucite chambers and sterile transfer techniques were made, but no difference in the subsequent growth of the tissues or in experimental results could be observed.

In cases where a preparation was to be used for more than one day, the seal was broken to permit entrance of a small air bubble into the lucite chamber; and the Holtfreter Solution was changed just before starting the experiments of the following day. Meanwhile, the lucite chamber containing the culture was kept in the refrigerator. Thus, in some cases it was possible to use the same tissue for experiments extending over 3–4 days.

Other conditions

As is general practice in tissue culture work, three drops of 1% Phenol Red solution were added to each 500 cm^3 of Holtfreter Solution, which displayed a pH of 7.2–7.4. The dye gave no discernible toxic effects and served as an excellent indicator of the condition of the culture. It was particularly useful in cases where a culture was kept for several days. Whenever there was indication of mounting acidity the solution was changed at once.

An initial series of experiments was performed before the thermally regulated chamber (described by Marsland, '50) became available. In this room-temperature series, however, the maximum variation during any one experiment was $\pm 1°C.$; and the total range of variation in the laboratory temperature during these experiments was between 23°–26°C. The temperature control chamber made it possible to perform experiments at various temperatures ranging between 5° and 40°C. and in these experiments the temperature variations produced very striking effects.

The pressures used in the experiments ranged up to 16,000 lbs./in^2. The pressure increases were achieved at the rate of 2000 lbs. per second and the decompressions were almost instantaneous.

Control cultures were set up for each series of experiments. These were contained in lucite chambers identical to those used in the pressure bomb and the treatment was identical except that the control preparations were kept on the stage of a microscope instead of in the pressure bomb. Generally speaking, the rate of beat in the control tissues remained quite constant (average variation $\pm 5\%$ throughout a day) as judged by periodic readings taken approximately every half hour. Each preparation tended to assume its own spontaneous rate of beat, and the variations in spontaneous rates usually fell between 30 and 60 beats per minute at room temperature. An occasional preparation was found in which the initial rate was exceptionally high (80 to 110). For the combined temperature-pressure experiments, a series of readings was made at each temperature before and after the application of pressure, which was standardized at 4000 lbs./in^2.

RESULTS

Pressure effects at room temperature

The routine procedure in these experiments was to observe the cardiac explant in the microscope-pressure chamber, ascertaining the spontaneous rate of beat, timed with a stopwatch repeatedly for about 20 minutes. Then the pressure was raised in steps of 2000 lbs./in.^{2}, maintaining each pressure level for 20–30 minutes. During this period the material was kept under continuous observation and the beat was timed at 1–5 minute intervals.

This type of experiment gave very uniform results. These are shown graphically in figure 1, a typical record selected from a total of 10 experiments.

Generally speaking, each abrupt rise of 2000 lbs./in., in the range up to 8,000 lbs./in.^{2}, resulted in a very large and immediate increase in the rate of beat. In many cases these increases exceeded 100% and they tended to reach a maximum in preparations having relatively slow (30–40 beats per minute) initial spontaneous rates. In fact, the records

show two preparations in which the initial rates of beat were exceptionally high (100 and 110 beats per minute) and in each case scarcely any acceleration was obtained when the usual compression procedures were followed. However, the same preparations used in later experiments, after the rates had slowed to 50 and 60, respectively, displayed typical compression acceleration effects. Maximum acceleration rates

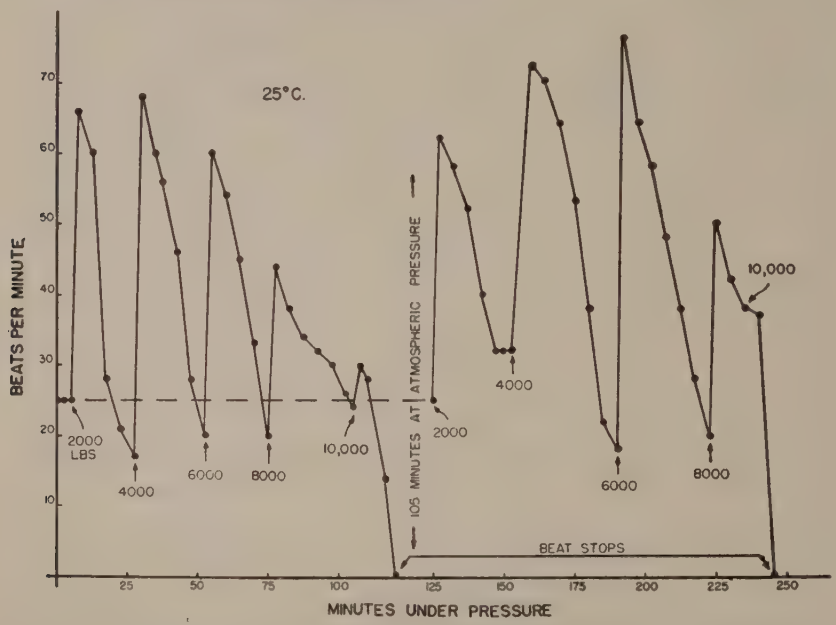

Fig. 1 The effects of maintained increments of pressure on the cardiac rate. Typical experiment at 25°C.

were usually obtained when the pressure level reached 4000 lbs./in.^{2}, although in a few particular cases the maximum was at 6000 lbs.

Following the rapid acceleration, which climbs to a peak within the first two minutes of the compression period, the rate of beat declines steadily (fig. 1). At pressure levels below 10,000 lbs./in.^{2} this deceleration is relatively slow, so that 15–25 minutes are required before the rate of beat returns to the initial value. However, at and above 10,000

lbs. the cardiac rate drops off very rapidly and within 5 minutes the heart fragment stops beating completely. In fact, if the tissue is suddenly exposed to a compression of 16,000 lbs. /in.^{2}, the accelerating effect of the pressure is scarcely perceptible and the beat comes to a halt within one minute.

Decompression effects

Decompressions of sufficient magnitude and speed (2000 lbs./in.^{2}/sec.) regularly resulted in a complete stopping of the beat or at least a drastic reduction in the rate of beat. This was generally true except at the lower range of temperatures (5°–13°C.) where the rate rapidly regained the precompression level as soon as decompression occurred.

In the room-temperature experiments the tendency for the beat to stop whenever the tissue was decompressed made it impossible to obtain a quantitative evaluation of the inhibitory effects. Generally the decompression heart-block endured for approximately 10 minutes, although in cases where the pressure level was high (above 10,000 lbs./in.^{2}) or had endured for a long time (more than 30 minutes) several hours elapsed before the tissue resumed beating at its pre-experimental rate.

Reversibility of the pressure effects

The cardiac tissue used in the experiments proved to be remarkably resistant to deterioration although exposed to long periods of compression and very drastic changes in the pressure level. An extreme case in point is one preparation which was carried through 4 successive days of experimentation. This tissue sustained elevated pressure for a total of more than 4 hours, and for one hour and 20 minutes the pressure level was above 8000 lbs./in.^{2}. Nevertheless, up to the end of the 4th day, the spontaneous rate of beat and the pressure responses of this preparation continued to be typical. However, it should be noted that at higher temperatures (35°C. and over) pressures above 4000 lbs./in.^{2} did in-

duce a marked irregularity of rhythm or a block of long duration.

The cardiac rate in relation to temperature

Since it has been shown (Brown, Johnson and Marsland, '42) that the sign of certain pressure effects may be changed according to the temperature at which the measurements are made, further experiments were undertaken to determine the temperature characteristics of these pressure results. First, however, it was necessary to determine the cardiac rate as a function of temperature, at atmospheric pressure.

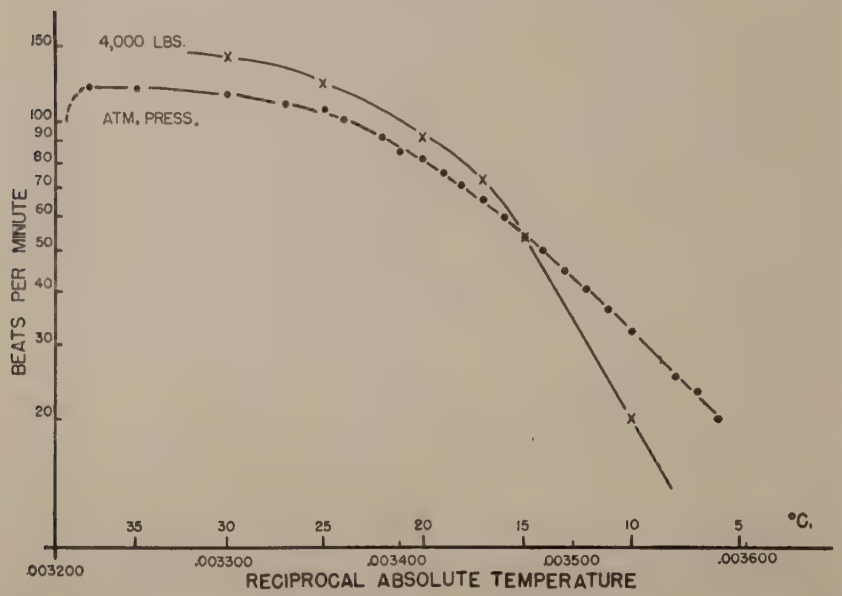

Fig. 2 Temperature-pressure effects on the cardiac rate. Each point is based on values derived from 6 different heart preparations. At atmospheric pressure it was necessary to select preparations displaying high initial rates of beat, in order to cover the whole range of temperature with the same culture. The initial rates of pressurized preparations were multiplied by factors which adjusted them to the level of the atmospheric preparations. For convenience, comparative centigrade values are given in the abscissa. It is to be noted that the sign of the pressure effect changes with temperature. Below 15°C. the rate is decreased; above 15°C. it is increased; whereas at about 15°C. pressure has little, if any, effect.

The results of these measurements are shown by the atmospheric pressure curve in figure 2, in which the log of the cardiac rate is plotted against the reciprocal of the absolute temperature, in the range between 5° and 38°C. At lower temperatures (as is characteristic of many biological rate processes) the log rate/temperature plot is linear. In other words, the cardiac rate increases exponentially with the absolute temperature in the range up to 12°C. Above 12°C., however, the rate of increase falls away markedly. Finally, at 38°C., the cardiac rate rapidly drops to zero, and once stopped at high temperature, the beating of the heart can seldom be restored.

Pressure effects in relation to temperature

These experiments were performed at 5° intervals in the range between 5° and 35°C. The standardized pressure treatment at each temperature was 4000 lbs./in.^{2}. This pressure was selected because it gave maximal effects in the room temperature experiments. Sometimes the same cardiac preparation was carried throughout the entire series of temperature changes, and a minimum of 6 preparations were utilized in obtaining each measurement. Whenever the temperature was changed, the cardiac tissue was left in the chamber at atmospheric pressure for two hours. This allowed ample time for complete temperature equilibration, which was checked by control experiments in which a thermometer was introduced directly into the pressure chamber, with the bulb not more than 0.5 mm distant from the tissue. In each case the beat rate was timed immediately before, and two minutes after, the pressure was established; and another reading was taken two minutes after decompression. The total compression time for each measurement was usually not more than 4 minutes.

It is clear from these experiments (see fig. 2) that the effects of pressure on the cardiac rate are different, both as to sign and magnitude, according to the temperature chosen for the observations. In fact, it is possible to spe-

cify three ranges of temperature in which the pressure results are different (figs. 2 and 3 A). (1) At lower temperatures (below 12°C.), in the range where the log rate/temperature curve retains its exponential character, the application of pressure produces a very definite retardation in the rate of beat; (2) at intermediate temperatures (14–16°C.), in the region where the log rate/temperature curve first begins to

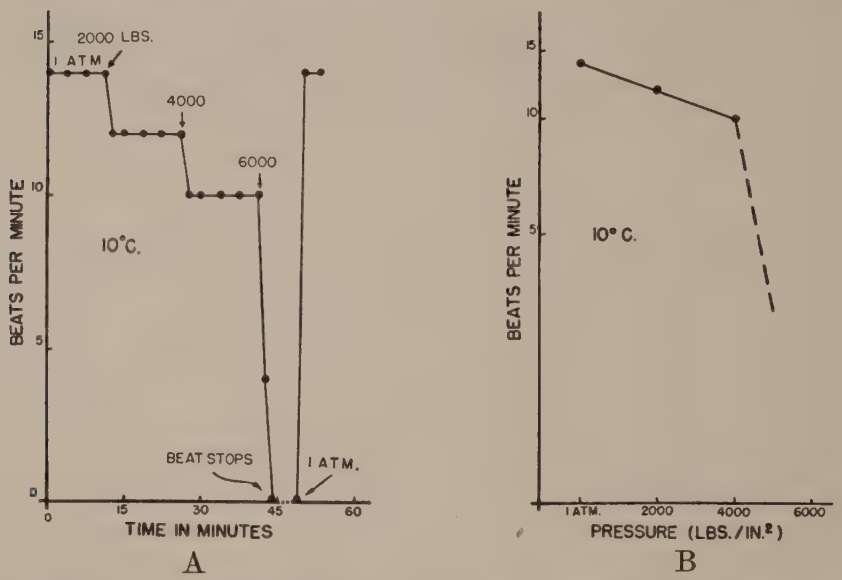

Fig. 3 A The effects of maintained increments of pressure on the cardiac rate. Typical experiment at 10°C.

B A replot of the data of figure 3 A as log rate/pressure.

fall off from linearity, pressure has very little effect upon the rate of beat; (3) at higher temperatures (above 16°C.), where the log rate/temperature curve rapidly diverges from linearity, pressure induces a marked acceleration in the rate of beat.

DISCUSSION

Many points of similarity are found when these experimental results are compared with the observations of earlier workers, who studied the effects of pressure on certain

other biological rate processes. Working at relatively high temperatures, for example, Edwards and Cattell ('28) observed the accelerating effects of high pressure on the rate of beat of the intact adult hearts of the frog and turtle; and Pease and Kitching ('39) reported the very striking, though transitory, speeding up of the ciliary beat when fragments of the gill of a mussel were exposed to hydrostatic pressures of similar magnitude. However, Brown, Johnson and Marsland ('42), studying the intensity of bacterial luminescence, were the first to point out that the sign of the pressure effect changes at lower temperatures.

Pease and Kitching ('39) proposed that two pressure-sensitive rate-controlling processes must be involved in the mediation of the pressure effects upon the ciliary beat. The first process was assumed to be dominant in determining the rapid acceleration which occurs (at higher temperatures) whenever high pressure is applied; whereas the second process was indicated by the declining rate which occurs when the pressure is maintained. However, this proposal fails to account for the observation that no pressure acceleration occurs at low temperature. To correlate all the pressure-temperature effects on the cardiac rate and to relate these results to the bioluminescence studies of Brown, Johnson and Marsland ('42), it seems necessary to postulate at least three rate controlling processes for cardiac tissue. Indeed, when the data of figure 3 A are plotted as log rate/pressure (fig. 3 B), the straightness of the line agrees with the idea of a single rate limiting reaction (Glasstone, Laidler and Eyring, '41), such as that of a rate limiting enzyme with a volume increase of activation ($\triangle V^*$) of, in this case, about 25 cm^3/mole.[3] The sudden drop at 6000 lbs./$in.^2$ is of such great magnitude that it may well represent (on theoretical grounds) a sudden change in state.

[3] We are pleased to acknowledge our indebtedness to Dr. Frank H. Johnson, Princeton University, for this calculation and for other improvements in the manuscript.

The limited data of this paper would justify only a very tentative formulation of the rate controlling reactions of cardiac tissue. The following reactions, on the other hand, provide at least a qualitative (though extremely simplified) explanation for all the observations; and they may be useful from the viewpoint of further experimentation:

$$\text{I. Precursory substances} \xrightarrow[\text{active enzyme}]{+\,\triangle V^*} \text{excitatory substances}$$

$$\text{II. Active enzyme} \underset{-\,\triangle V}{\overset{+\,\triangle V}{\rightleftharpoons}} \text{thermally inactivated enzyme}$$

$$\text{III. Thermally inactivated enzyme} \xrightarrow[-\,\triangle V^*]{\text{slow; irreversible}} \text{denatured enzyme}$$

According to this tentative scheme the cardiac rate in isolated heart fragments would be determined *directly* by reaction I, a metabolic process initiated after each beat, which builds up excitatory substance until the concentration is adequate to precipitate the next beat. At low temperatures (under 12°C.) apparently, reactions II and III are not significant. That is to say, thermal inactivation or denaturation of the enzyme component of the excitatory reaction does not come into play. At low temperatures, therefore, the only effect of pressure is to inhibit reaction I, as would be expected on the assumption that a volume increase ($+\,\triangle V^*$) is characteristic of the reaction. Moreover, in the lower temperature range, in the absence of any thermal inactivation of the enzyme component, an exponential acceleration of reaction I would be expected with rising temperature (fig. 2).

In the intermediate temperature range (12°–16°C.) reaction II, a reversible thermal inactivation of the enzyme component, begins to be significant, as is indicated by the fact that the log rate/temperature curve shows its first small departure from linearity (fig. 2). In this range, therefore, pressure would be expected to have a two-fold effect: (1) a retardation effect (inhibition of reaction I), and (2) an accelerating effect (inhibition of reaction II, which tends to

restore a more effective concentration of the enzyme component). In this range, therefore, pressure has very little, if any, net effect upon the cardiac rate. In short, there is a balanced cancellation of the retarding and accelerating actions of the pressure, at intermediate temperature.

At higher temperatures (16°–30°C.), the dominant immediate effect of pressure would seem to be a reversal of reaction II, the thermal inactivation of the enzyme component. This reversible reaction is assumed to have a relatively high positive $\triangle V$ value, in which case the acceleration resulting from a restoration of active enzyme would more than compensate for the direct retardation of reaction I by pressure. In other words, the main immediate action of high pressure acting at higher temperatures is a tendency to restore the exponential character of the log rate/temperature curve. Such a restoration can never be complete, however, because simultaneously, pressure exerts a direct inhibiting influence on reaction I.

In accordance with the suggestion of Pease and Kitching ('39), reaction III accounts for the fact that the high-temperature pressure accelerations do not endure (see fig. 1). This irreversible (or very slowly reversible) thermal denaturation of the enzyme component does not appear at lower temperatures (below 20°C.) when the cardiac rate remains steady even when a compression is maintained. At and above 25°C., however, reaction III appears to have an increasingly important influence on the effective concentration of active enzyme. When the pressure is maintained at such temperatures, the initial acceleration — which is mediated rapidly through reaction II — slowly disappears, and indeed the rate continues to fall even below the precompression value (see fig. 1). Moreover, this decaying of the cardiac rate during the compression period proceeds much more rapidly at higher pressures, as would be expected on the assumption that reaction III is characterized by a high negative $\triangle V^*$ value. And finally, the irreversible stopping of the heart which occurs with high temperature (38°C.) alone, or with high tempera-

ture and pressure (35°C./4000 lbs.) indicates that any restoration of the active enzyme component after the terminal stage (reaction III) of denaturation, must occur slowly, if at all.

The foregoing three-reaction system seems to provide a fairly simple qualitative explanation of temperature-pressure observations, but the data are not sufficient for extensive quantitative treatment. However, a calculation of the apparent $\triangle H^*$, at atmospheric pressure and at 4000 lbs. (from the low temperature data of fig. 2) yields values of 18,500 cal./mol. and 33,800 cal./mol. respectively. These values are very similar to and in accordance with the findings on bacterial luminescence. Many difficulties are encountered in setting up and maintaining culture preparations of cardiac tissue under standardized conditions, and the range of variation in cardiac rate is somewhat limited. Therefore, we plan to continue the experiments by studying the effects of temperature and pressure on the rate of beat of cilia in the clam's gill, which appears to be a much more favorable material.

SUMMARY

1. The rate of beat of tissue culture preparations of the tadpole (R. pipiens) heart has been studied as a function of both pressure and temperature.

2. Both the sign and the magnitude of the pressure (4000 lbs./in.^{2}) effect on the cardiac rate vary according to the temperature at which the experiments are performed. As to sign, below 14°–16°C. the rate is decreased, above 14°–16° it is increased; whereas in this critical range of temperature, pressure has little, if any, effect upon the cardiac rate.

3. Increases in the cardiac rate induced by sustained pressure at higher temperatures do not persist. The rate gradually falls away, usually dropping below the atmospheric level within approximately 10 minutes.

4. The data are interpreted with the aid of the Glasstone-Laidler-Eyring Theory of Rate Processes. The main rate regulating reaction appears to have a positive $\triangle V^*$ value,

and is inhibited by pressure throughout the whole range of temperature. At higher temperatures, however, the enzyme system seems to display a rapid reversible inactivation, with a positive $\triangle V$ value, and a slow irreversible denaturation, with a negative $\triangle V^*$ value. Therefore, the initial effect of pressure in the higher temperature range is to increase the rate, whereas the final effect is to lower it.

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EFFECTS OF ULTRASOUND ON CELL STRUCTURE[1]

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FOUR FIGURES

INTRODUCTION

Since the development of technics for the production of ultrasound in liquids over 30 years ago, it has often been found that ultrasonic waves can produce injury or complete destruction of living cells. Observations have been made on a variety of plant and animal cells. Among the effects noted have been changes of permeability to Na and K hydroxides, changes in protoplasmic viscosity, displacement and disintegration of intracellular structures, coagulation or swelling of protoplasm, dispersion of cell contents, and complete destruction of the cell. However, no observations on the time sequence of events which take place during exposure to ultrasound appear to have been recorded. This report is an attempt to supply such information based on preliminary studies of the mechanisms involved in the destructive processes.

MATERIAL AND METHODS

The organisms studied were two species of the filamentous alga Spirogyra, the water plant Elodea (Anacharis) canadensis, the yeast Saccharomyces cerevisiae, human and chicken erythrocytes, and a Holotricha infusorian (Holophrya, Ehrenberg).

The source of ultrasound (fig. 1) was a commercial generator[2] which produced a small vertical beam at frequencies

[1] The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department.

[2] Ultrasonorator, Crystal Research Laboratory, Hartford, Connecticut.

of 400, 700, 1000 or 1500 kilocycles (wave lengths in water 3.75, 2.14, 1.50, 1.00 mm) at an intensity sufficient to produce marked gas bubble formation in the liquid over the quartz crystal and a liquid column extending several inches above the surface of the liquid. The transformer oil in which the

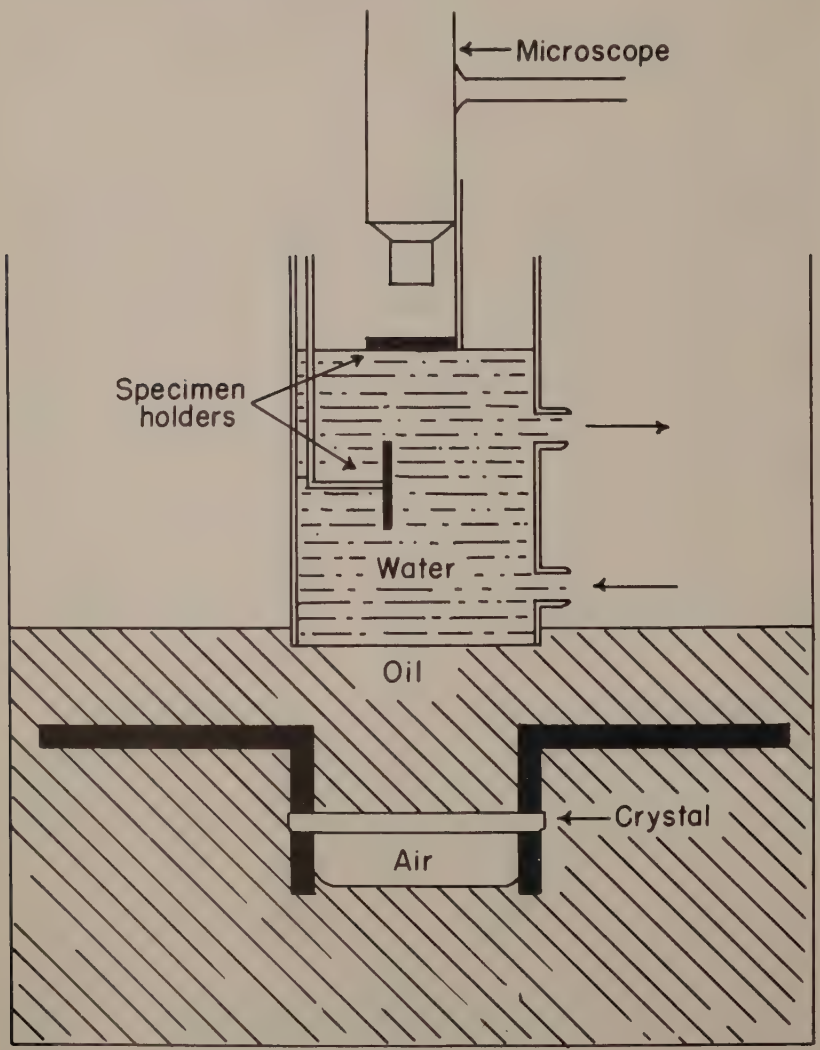

Fig. 1 Schematic diagram of apparatus for exposure of small organisms to ultrasound.

crystal was immersed was cooled by circulation through a refrigerated bath to maintain its temperature and that of the specimens below 30°C.

The preparations were suspended in a glass cylinder of water placed vertically in the oil over the crystal so that the sound beam passed up through it and was reflected by the water-air interface or by a very thin glass membrane placed at the surface. The organisms were mounted in a cage of glass hairs on a glass slide or between a pair of cover glasses. When desired, a small cover glass cell could be mounted horizontally at or just below the water surface on a microscope rack for direct observation during exposure.

The extreme difficulty of measuring sound intensity at the frequencies used constitutes a barrier, for the time being, to quantitative measurement. In addition, relatively small changes in the physical arrangement of the apparatus may have a marked effect on the mechanical impedance of the system and thus on the strength of the ultrasound field resulting from a given electrical power input. The electric power readings were used, therefore, only for very rough duplication of experimental conditions and of the sound intensity. As a rule, the settings were adjusted to produce destructive effects over a period of several minutes, thus making it convenient to follow the sequence of events. In addition to direct observation, a few qualitative tests were used to follow changes in cell permeability and dye absorption. The cells were plasmolyzed with sucrose or potassium nitrate or were immersed in dilute neutral red or methylene blue.

RESULTS

The observations reported here were made repeatedly and were readily reproducible. No qualitative difference among the effects of different frequencies was found. With direct observation during exposure, no gas bubbles were seen in or close to the injured cells. The cellular changes occurred independently of the orientation of the cell with respect to the direction of the sound propagation.

Spirogyra. In previous observations (Wood and Loomis, '27; Schmitt and Uhlemeyer, '29; Harvey, '30), an increased permeability to Na and K hydroxide, disruption of cell walls, and a dispersion of chlorophyl have been noted. In our experiments two species were used. In the first, the cells were 66 micra thick, with 4 chloroplasts per cell (fig. 2a); in the second, the cells were 30 micra thick, with three chloroplasts per cell. Both species reacted to exposure in the same way and in the following sequence: (a) There was a cessation of flow of the protoplasmic threads which support the nucleus in the middle of the cell; the threads flowed toward the cell wall protoplasm and the nucleus was displaced toward the side of the cell (fig. 2b). (b) The protoplasm became swollen; the chloroplasts shifted toward the center of the cell, contracted and fused together, forming droplets (fig. 2, c and d). (c) The nucleus swelled and usually dissolved in the cell sap (fig. 2 d) but occasionally coagulated. (d) As the protoplasm coagulated and was separated from the cell wall, the chloroplasts remained liquid. (e) The chloroplasts coagulated and the entire cell became a coagulated mass (fig. 2 e). (f) The protoplasm and chloroplasts disintegrated and became a suspension of minute granules (fig. 2 f). The cell interior cleared and the chlorophyl was spread uniformly throughout the cell. Sometimes the green color disappeared. Starch grains, if present in chloroplasts, were torn into small pieces. Recovery of the cells on removal from the sound field occurred if only the earliest changes had taken place, i.e., the beginnings of chloroplast displacement.

The coagulation of the protoplasm was accompanied by a loss of semipermeability and cell turgor. Normal cells were markedly plasmolyzed by immersion in 0.5 M KNO_3. However, if the cells had first been exposed to ultrasound and showed a considerable displacement of chloroplasts, the plasmolysis was very slight. If normal cells were plasmolyzed by 0.7 M sucrose, allowed to reach equilibrium, and then exposed to ultrasound, the volume of the cell sap decreased further. This indicates that the permeability to KNO_3 and

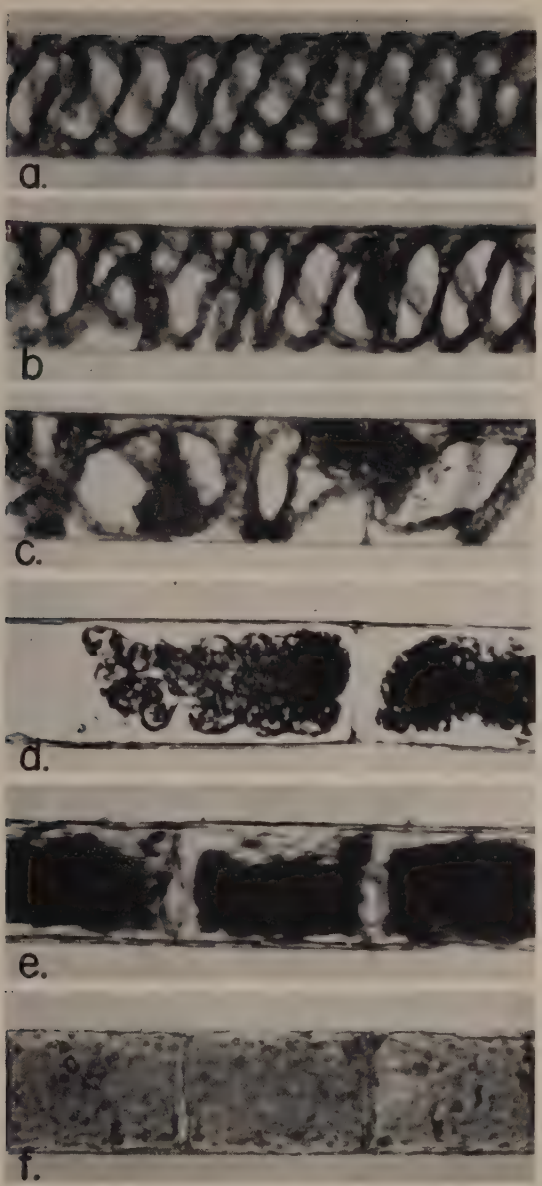

Fig. 2 Spirogyra. Magnification of all photographs, ×260.

a. Normal cells. Nucleus suspended in center of cell.
b. Cells affected by ultrasound. Beginning displacement of chloroplasts. Nucleus displaced toward cell wall.
c. Displacement of chloroplasts continues. Beginning of fusion.
d. Fusion and droplet formation by chloroplasts.
e. Coagulation of entire protoplasm and cell death.
f. Post-mortem dispersion of coagulated cell contents. Dispersed material fills entire cell. Cell interior clears up.

substances of the cell sap was increased by ultrasound. The form of the protoplasm plasmolyzed by 0.7 M sucrose was spheroidal; on exposure to ultrasound the protoplasmic surface became irregular. In dying plasmolyzed cells, coagulation of the peripheral part of the protoplasm was frequently seen, with the formation of so-called tonoplasts taking place prior to completion of the coagulation process.

Elodea. Exposure of this organism can result in violent intracellular motion, emulsification of cell contents and complete cell destruction (Harvey, Harvey and Loomis, '28; Harvey, '30). The following sequence of events was seen on direct microscopic observation of Elodea leaves exposed to ultrasound (fig. 3): (a) The rotary motion of protoplasm, induced by cutting off the leaf, ceased. In those cells near the leaf nerve where the viscosity of the protoplasm appears to be relatively low, the motion of the cell sap produced by the ultrasound caused pieces of protoplasm containing chloroplasts to separate. These pieces at once became spherical and rotated about their axes. A similar movement has been observed by Harvey ('30). (b) The chloroplasts, which in intact cells have a disc-like form and are uniformly distributed throughout the cell (fig. 3 a), became globular, were all displaced toward one side of the cell and occasionally fused together (fig. 3 b). (c) The nucleus swelled up. (d) The nucleus dissolved or coagulated. The protoplasm coagulated and separated from the cell wall (fig. 3 c). (e) The chloroplasts coagulated and post-mortem changes began. (f) The coagulated protoplasm and chloroplasts disintegrated to form a suspension of minute granules which showed Brownian movement (fig. 3 d). The suspension was so fine that no granules were visible and the cells appeared empty (fig. 3 e). However, starch grains, when present, were broken into small pieces but did not dissolve (fig. 3 e). No destruction of the cell walls occurred. None of the observed changes were spontaneously reversible.

Saccharomyces cerevisiae (compressed yeast, National Yeast Corporation). Yeast cells are destroyed by ultrasound

Fig. 3 Elodea leaf. Magnification of all photographs, × 487.5

a. Normal cells of upper layer.
b. First phase of ultrasound effect. Chloroplasts become globular and are displaced toward one side of cell.
c. Protoplasm and chloroplasts coagulated and separated from cell walls. Cell death.
d. Post-mortem dispersion of coagulated cell contents. Entire cell filled with minute granules. Starch grains remain.
e. The dispersion of cell contents is so complete that the cells appear empty. Starch grains are partly disrupted.

(Beckwith and Olson, '31). With this organism the sequence of events was as follows: (a) The first visible change was the appearance of large granules in the protoplasm with the spherical vacuoles becoming irregular in shape. (b) The protoplasm congealed, contracted, and drew away from the cell walls. Post-mortem disintegration of cell contents occurred as in other plant cells.

Human erythrocytes. Observations on the exposure of mammalian red blood cells to both audible and ultrasound have often been made. The hemolysis observed (Wood and Loomis, '27; Johnson, '29; Chambers and Gaines, '32; Sibuya, '36) has been shown not to be osmotic in nature (Cerny, Leichti and Wilbrandt, '42). In these experiments oxalated human blood diluted 1:10 in 0.95% NaCl containing phosphate buffer at pH 7.3 was used. On exposure to ultrasound, the cells changed steadily from their normal disc shape to a bell shape, then to a spherical form and were finally hemolyzed. The stroma disintegrated completely.

Chicken erythrocytes. Nucleated red cells have also been studied (Harvey, '30; Chambers and Harvey, '31; Morelli and Reggiani, '41; Kazahara, Naruse and Sakiyama, '37). Hemolysis has been observed without destruction of the nuclei. For our experiments, heparinized blood of a one-month-old chicken was diluted 1:5 in 0.95% NaCl. Normal cells are oval in shape with central nuclei of variable form (fig. 4 a). The nuclei have somewhat indistinct outlines, an emulsion structure and a refractive index greater than that of the cytoplasm. When the intensity of the ultrasound was such that complete hemolysis occurred in about 7 minutes, a three-minute exposure produced swelling of the nucleus and its dissolution in the cytoplasm in 90% of the cells. (fig. 4, b, c, d). In the remainder, the nucleus became spherical and coagulated (fig. 4, e, f, i, j). Before hemolysis, the cells took a spherical form because of complete liquefaction of the membrane and border hoop. After hemolysis, the cells disintegrated completely, leaving nuclei behind in those cells in which nuclear coagulation had occurred (fig. 4, g and h). The stroma remained

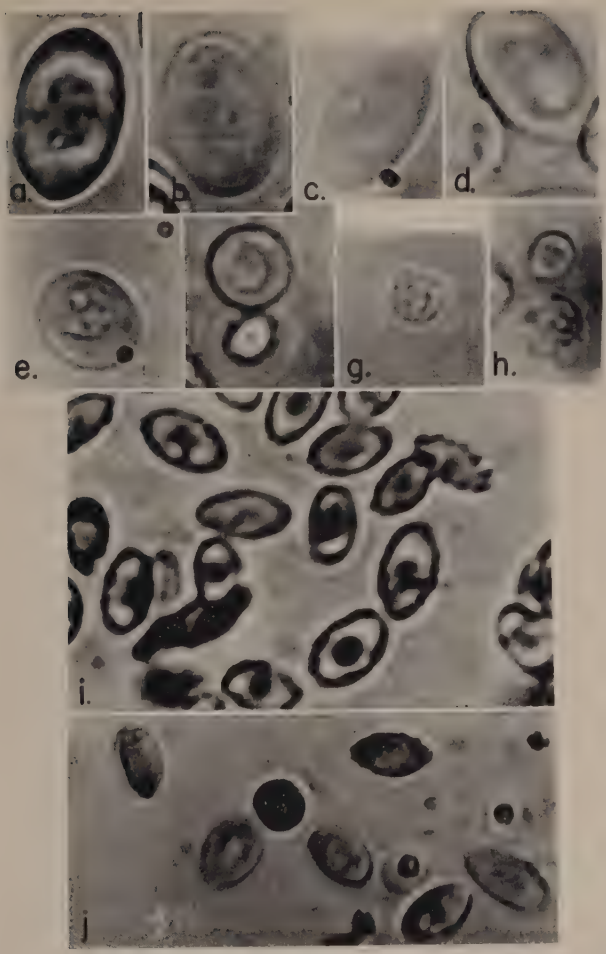

Fig. 4 Chicken erythrocytes. Magnification of a to h, ×2175; of i, × 1050; and of J, × 900.

a. Normal cell. Nucleus has irregular form and indistinct boundary.

b, c and d. Erythrocytes affected by very intense ultrasound. Nuclei dissolved in cytoplasm; cells are denucleated.

e. Some cells take a globular form (complete liquefaction of the cell membrane and the border hoop). Nucleus shrinks and coagulates.

f. Spherical cells containing coagulated nuclei (above) and hemolyzed (below); indistinct stroma visible after hemolysis.

g and h. Stroma dispersed; coagulated nuclei remain.

i. Chicken erythrocytes exposed at relatively low intensity. In most cells nuclei are coagulated. Few cells show an irregular border (beginning of liquefaction of the cell membrane and border hoop).

j. Effect of exposure at relatively high intensity. Four cells are denucleated. One denucleated cell and two cells with nuclei have a spherical form. Nuclei are coagulated.

for a very short time (fig. 4 f), then disintegrated. When the intensity of the ultrasound was such that complete hemolysis required only two or three minutes or as much as 20 to 30 minutes, almost all of the cells showed coagulated spherical nuclei which remained after destruction of the stroma (fig. 4 i).

Holophrya. Protozoa have also been frequently studied. Amoeba and paramecia are immobilized and torn apart (Wood and Loomis, '27; Harvey, Harvey and Loomis, '28; Schmitt, Olson and Johnson, '28; Chernuk, '39). The cells of the species of Holophrya used were 40 to 55 micra long, 30 to 35 micra wide, having a cytostome without any ciliary ring, a cytopharynx without trichites, few food vacuoles, a large contractile vacuole, and a spherical macronucleus. Exposure of this organism to relatively intense ultrasound produced (a) an initial increase in motility, followed by a gradual cessation of motion; (b) the cells swelled to approximately 55 to 88 by 30 to 45 micra, often thereafter becoming spherical. The food and contractile vacuoles disappeared and new large vacuoles appeared, which sometimes flowed together. (c) The macronucleus became swollen and often dissolved in the cytoplasm. The cytopharynx and trichocyst bulbs also swelled and became round. Sometimes the cytopharynx was forced to the surface and formed a tentacle-like or circular projection. (d) The cytoplasm, trichocyst bulbs and macronucleus (if not already dissolved) congealed and shrank. The entire cell became filled with coagulation granules. The cytostome and cytopharynx disappeared, leaving a small cavity in the surface. Small pieces of the coagulated material were gradually torn away until the whole cell was dispersed in the surrounding medium.

Vital staining. Most normal cells, if immersed in dilute (.001% to .01%) neutral red or methylene blue solution, show color only in the sap of plant cells or in vacuoles and granules of animal cells. The rate at which the dye is taken up is a rough index of permeability to the dye. When a mixture of these two dyes is used, the structures mentioned become red. The nucleus and cytoplasm of most moribund cells become violet

in the mixture, while the completely coagulated protoplasm becomes blue. Bauer ('27) has attributed this sequence of color changes to a decreasing pH of the cell contents. In our experiments, 0.5% NaCl was added to the solutions to prevent adsorption of the dye by the cell walls.

In Spirogyra, dye accumulation occurred much more rapidly in cells injured by ultrasound than in normal cells although the final color intensity was the same in both cases. The protoplasm and chloroplasts did not absorb the dye until coagulation had occurred. After dispersal of the cell contents, dye accumulated as strongly as in the coagulated protoplasm. In a dye mixture at pH 7, the sap of the normal cells became reddish. At the beginning of coagulation, the protoplasm took a violet stain and after complete coagulation, a blue stain along with the chloroplasts. After dispersion of the coagulated material had occurred, the cell contents stained blue, but dye accumulation no longer occurred after the process was complete. A similar sequence was observed with Elodea.

With yeast cells which normally take up no dye from a mixture of pH 4.8, absorption of neutral red by the cell sap occurred after an exposure to rather intense ultrasound which was so short as to produce no visible changes. The granules which later appeared in the protoplasm took an intense pink stain compared with a weak pink in the protoplasm and cell sap. Again, the coagulating and coagulated protoplasm became violet and blue, respectively. Erythrocytes took up a stain only immediately before hemolysis. The coagulated Holophrya was stained violet.

Effects of heat. Some of the organisms were killed by immersion in hot water and the cellular changes were observed for comparison with the effects of ultrasound. Spirogyra and Elodea exposed to 50°C for 15 minutes showed no swelling of the nucleus or protoplasm; no displacement of chloroplasts or formation of droplets occurred. The chloroplasts coagulated before the nucleus or cytoplasm. No dispersion of cell contents occurred even after continued exposure for several

hours. When starch grains were present in the chloroplasts, they swelled up and often filled the entire cell.

When Spirogyra was exposed to ultrasound in water at 50°C for 10 to 15 minutes, the chloroplasts were very slightly displaced but were coagulated by the heat. After a cell had been killed, its contents were dispersed. However, if the cells had first been boiled so as to coagulate the proteins, exposure to ultrasound did not produce dissolution of the cell contents.

If chicken erythrocytes were immersed for 5 minutes in water at 57°C they became irregular in form, often with droplet-like projections. After 20 minutes' exposure, they became spherical and the refractive index of the cytoplasm became greater than that of the nucleus. The nucleus finally coagulated, and hemolysis occurred but both stroma and nucleus remained.

Infusoria immersed in water at 44°C became nearly spherical within one minute. The vacuoles disappeared but the macronucleus did not swell. After three minutes the nucleus and protoplasm coagulated. No dispersion occurred even after an hour. The pellicle sometimes separated from the cytoplasm but did not disappear.

DISCUSSION

The effects of exposure to ultrasound appear, in general, to follow this course: displacement of nucleus and chloroplasts in plant cells, fusion of chloroplasts and their conversion into droplets, coagulation of the nucleus or its dissolution in the cytoplasm, coagulation of the cell contents followed by dispersion. These changes, at least in Spirogyra, are the same as those produced by other mechanical forces, such as blows (Lepeschkin, '27). Heat appears to produce only coagulation and shrinkage of chloroplasts, nucleus and cytoplasm. No displacement of chloroplasts occurs. The fact that the changes produced by heat are different from those produced by ultrasound and that heat does not potentiate the effects of ultrasound, makes it unlikely that thermal effects

are important in the changes brought about by exposure to ultrasound. It is also quite unlikely that ultrasound produces any significant local heating of cell structures. When cell death processes proceed slowly under the influence of any of a number of agents, swelling of the protoplasm and liquefaction of cell structures often occur. Dissolution of the nucleus in the cytoplasm or cell sap of plants is also a common phenomenon as is the formation of vacuoles after the absorption of water in animal cells (Lepeschkin, '27, '37) the increase in permeability, and changes in vital staining reactions. However, the post-mortem disintegration and dispersion of coagulated material appears to be a specific effect of ultrasound.

The extent of the changes described here is presumably limited by the available sound field intensity and by the duration of exposure. The findings by some investigators of complete destruction including even plant cell walls may well be the result of the application of very intense fields for long times, possibly accompanied by violent circulation of the liquid medium in which the cells were immersed.

SUMMARY AND CONCLUSIONS

1. The effects of ultrasound at frequencies of 400, 700 and 1000 kilocycles have been investigated on the following kinds of cell: Spirogyra, Elodea (Anacharis) canadensis; Saccharomyces cerevisiae; human and chicken erythrocytes; and an infusorian (Holophrya).

2. Exposure to ultrasound causes swelling of nucleus and cytoplasm, displacement and fusion of chloroplasts (when present), their conversion into droplets, and dissolution of the nucleus in the protoplasm or cell sap. Occasionally the nucleus coagulates. The absorbed water may be separated again in the form of vacuoles. The final phase is complete coagulation of the living matter followed by dispersion of the cell contents or hemolysis. In these experiments no disruption of plant cell walls was produced.

3. Vital staining shows dye absorption by protoplasm and nucleus as a rule only during and after coagulation or, in red cells, just before hemolysis.

4. In Spirogyra, ultrasound appears to increase the permeability of protoplasm to potassium nitrate and substances of the cell sap.

5. The sequence of changes produced by ultrasound differs markedly from that produced by immersion in hot water.

6. Only the slightest changes in cell structure, produced by exposure to ultrasound, are spontaneously reversible.

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REPETITIVE DISCHARGES OF MOTOR FIBERS CAUSED BY A SINGLE IMPULSE IN GIANT FIBERS OF THE CRAYFISH

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SIX FIGURES

INTRODUCTION

In previous publications (Wiersma, '47, '49) it has been pointed out that the transmission across the central synapses of the crayfish shows differences which are correlated to their function. Transmission may be either uni- or bidirectional, and may be one-to-one or in need of facilitation. The present paper will describe still another way of transmission, namely, a one-to-many type, in which a single giant fiber impulse can trigger a volley of impulses in a single efferent fiber.

The efferent fibers, which show this phenomenon, also show spontaneous activity. The relation between this activity and the readiness of the fiber to give an afterdischarge has been studied both under normal circumstances and when the preparation was under the influence of drugs or ionic changes in the perfusion solution.

In all preparations the reactions in two different roots of abdominal ganglia have been studied. The results have thrown light on the simultaneous reactions of paired roots of one ganglion.

Because of the fact that the phenomena appear in their simplest form in the second roots of the anterior (second and third) abdominal ganglia, these have been especially studied. The action potentials of the first and third roots of these

ganglia, as well as all roots of more posterior ganglia, have been compared with these.

The first root primarily innervates the swimmerets, the second root the extensor musculature, and the third the flexor musculature. First and second roots carry also a large number of sensory fibers, for, respectively, the ventral and dorsal parts of the segments, whereas the third ones are perhaps exclusively efferent in nature and much thinner.

METHODS

The giant fibers of *Cambarus clarkii Girard* were prepared in the oesophageal commissures, as single fibers. Special care was taken that they were not accompanied by any of the smaller fibers surrounding them. All other fibers were cut. Crossing of the impulses between the two medial fibers, which is possible in the brain (Wiersma and Turner, '51) was prevented by slight damage near the brain to the medials, when both were left intact, or by removal of one. The abdominal part of the cord was exposed in the usual manner (Wiersma, '47). The second roots and, in a number of experiments, the first as well, were prepared for a distance as long as the method of preparation allows. The resulting length is of the order of 10 mm.

With the use of platinum, micromanipulated electrodes, the isolated part of the giant fiber was stimulated with square single descending shocks of 0.5 milliseconds' duration. Leading off from the roots was performed by lifting the cut end on a micromanipulated electrode above the level of the perfusion fluid. Two roots were used at the same time, sharing a common electrode which was submerged in the perfusion fluid. After appropriate amplification, the potentials were led to a Du Mont double-beam cathode-ray oscillograph.

In the experiments in which drugs were added to the perfusion fluid, the latter was always 100 cm^3 in volume, and 0.5 or 1 cm^3 of the drug in an appropriate concentration was given, so that the final concentration was known.

RESULTS

A. Comparison between the second roots of one ganglion. The spontaneous discharge. When the left and right second roots of one ganglion are put on the electrodes, it is regularly found that in both there is a fiber with an intermediately-sized action potential which is spontaneously firing. The frequency of this firing varies in different preparations and changes from time to time in a given preparation. Especially when the animal has been freshly prepared and was in a lively condition, there are often periods of high activity alternating with ones in which the frequency is much lower.

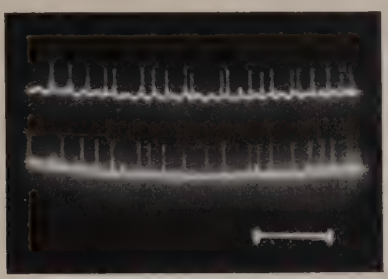


Fig. 1 Spontaneous activity in the second roots of the second abdominal ganglion. Top record: left root; bottom: right root. Frequency about 50 per second. Time shows 100 milliseconds.

In no case was complete silence of the second roots observed in a living preparation. The "basic" discharge rate is from 5 to 10 per second. During the high activity periods, the rate can become as high as 50 per second (fig. 1). Especially during such periods there is also evidence of the firing of a much smaller fiber. The latter is, however, much less regular (fig. 1) and may be absent during the "basic" discharge. Its relations have not been further studied.

In comparing the activity of the two roots of one ganglion, it is observed that the over-all frequency is usually very similar. The timing of the impulses in the roots, however, is not the same, but shows periods of synchronous discharges and periods during which the two sides are completely out of step. Synchronism is maintained for a small number of

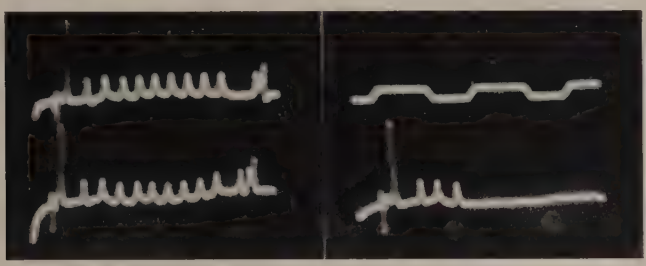
impulses (e.g., 4 or 5), after which for two or three impulses the two sides behave independently (fig. 1). From a study of a number of cases the impression was gained that both sides have their own automaticity, which is usually very similar, and that the level varies continuously. When both sides are "ready" about the same time for firing, the one that fires first causes the other to go off too, and will be pacemaker for the next few impulses. However, even during a synchronous period, the lead may shift to the other side, and during successive periods either side may have the lead at the start. Such periods of synchronizing have been obtained in all undamaged preparations.

The discharges on giant fiber stimulation. When a giant fiber is stimulated a discharge takes place in both second roots of one ganglion. Typically (as found often on medial giant fiber stimulation and leading off from the second roots of the third ganglion) such a discharge consists of two types of action potentials. It starts with a large spike, which is caused by a large fiber in the root, and this is followed by a row of action potentials of a medium-sized fiber. In form and shape the latter action potentials are identical with those of the spontaneously discharging axon in the root, and must therefore be considered as caused by the same axon.

In order to describe the phenomenon more precisely, it is necessary on the one hand to describe the different results obtained from different preparations in the same roots, and from the same preparation at different times, and on the other hand to compare the discharges of the two roots of one ganglion. In general, the first differences cover a much wider range than those of the second type.

Variations in the discharge of a second root are very great. In older preparations, though still alive, no discharge at all may be obtained in the second roots. This circumstance has been the reason that previously second root discharges have been found only occasionally (Wiersma, '47). When they were found, they occurred only after a considerable delay, and showed no repetition. An explanation of these findings lies in

the fact that older preparations were used, and that repetitive discharges will quickly disappear when the giant fibers are stimulated for any length of time, such stimulation being customary in the study of third root potentials. Even stimulation frequencies of as few as one per second will gradually reduce the number of repetitive discharges in each burst (fig. 2). With higher frequencies they soon disappear completely, only a single discharge remaining. A rest may at first more or less restore them, but eventually this procedure also becomes ineffective. All experiments to be described were therefore performed with a very low stimulation frequency (once every two



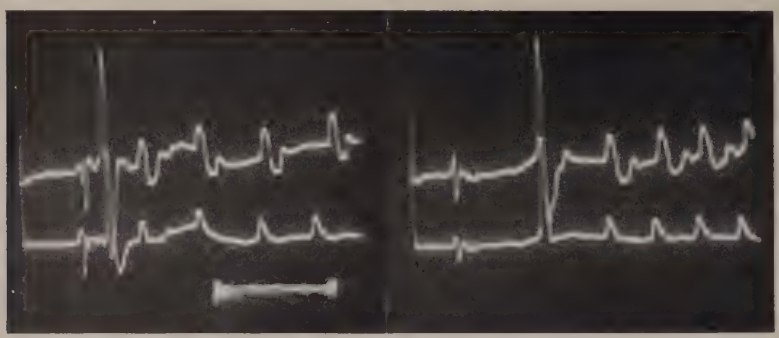
A B

Fig. 2 Repetitive activity of second roots of third abdominal ganglion on stimulation of left medial giant fiber with single shock. (A.) Fresh preparation. Top: right root; bottom: left root. (B.) Preparation after 30 seconds' stimulation at one per second. Top record: time in 1/60 seconds.

seconds) and frequent rests were given. Even so, the uncontrollable condition of the animal which obtained before preparation was undertaken had a profound influence on the results. Great variation is present with regard to (a) the occurrence of the large fiber potential, (b) the delay of the first impulse, and (c) the number of repeats. There is, furthermore, no obvious relation among these variables. In all preparations, there is a strong tendency toward conformity between successive stimuli in all three respects. The large potential typically occurs at the start of the discharge; however, it may also appear later in the discharge, or in other cases, repeat itself. Repetition is never so pronounced as in the case of the smaller fiber, and there are rarely more than

two large spikes present in any discharge of the anterior roots. A preparation which shows only one spike at the beginning will usually only change by losing the spike and will not show a delayed spike or repetition.

The delay of the first impulse is sometimes very pronounced. Figure 3 M shows a case in which the delay of the large potential, accompanied by a simultaneous firing of the smaller fiber, is about 6 milliseconds, a period of time which is by no means a record. In this case the delay took place on stimulation of medial giant fiber, whereas stimulation of the



L M

Fig. 3 Second root discharges of third abdominal ganglion on stimulation of lateral and medial giant fibers. L. Stimulation of left lateral giant fiber. Top record: right root; bottom: left root. Time, 10 milliseconds. M. Stimulation of right medial giant fiber. Notice long delay of impulse series, and higher frequency, once started.

lateral giant fiber caused in the same preparation a short delay (1.5 milliseconds) (fig. 3 L). The long delay on the medial giant fiber stimulation changed later to a short delay. When the large potential does not occur at the beginning of a discharge, but during it, its delay may become as long as 40 milliseconds. The delay of the first discharge of the smaller fiber is quite variable but most often from 5 to 10 milliseconds.

The number of repetitive discharges in the smaller fiber is very variable among different preparations, but is usually constant within plus or minus one within any series of trials. In some preparations it may exceed 10, though more often the number is smaller. In some it is only one or two repetitions

from the start. As mentioned, it is always possible to reduce the number by more frequent stimulation.

The resemblance between the two roots of one ganglion is on the whole very striking. The greatest difference occurs with regard to the fast fiber potential, when this is not located at the start of the discharge in one or both roots. The repetitive discharges in the smaller fibers are very often very similar and in the great majority of cases did not vary by more than one in number from each other. The two discharges may therefore be almost identical and only a closer study will reveal small time differences which are partially caused by the fact that the giant fiber impulse is homolateral for one side and not for the other (fig. 3).

Especially noticeable and found in all preparations which repeat more than three times is the high frequency of discharge reached by the smaller impulses. In longer discharges, it is always well above 200, and often exceeds 300 somewhat (fig. 2 A). When the number of discharges is reduced by more frequent stimulation, the discharge typically shortens, without much change in the frequency of the first impulses. This is illustrated by figure 2 B for one root, which was taken after a period of stimulation at the rate of one per second for a period of 30 seconds. The frequency is here of the order of 300 per second.

With regard to the first spike of a series, this may represent, depending on circumstances, one of three cases, (a) a pure large potential, (b) a combination of large and smaller, or (c) a small potential, as illustrated, respectively, by figures 3 L, 3 M and 6 A. The first two possibilities are sometimes difficult to distinguish from each other.

B. Comparison of discharges in the second roots of one ganglion on stimulation of different giant fibers. As stated, the discharges can be brought about by the stimulation of any of the giant fibers. Stimulation of the two lateral fibers will result in very similar responses, since on stimulation of one, the other is also always excited by the cross connections in the ganglia (Wiersma, '47). There thus remain the possibili-

ties of comparing the effects of the two medials and of a lateral with a medial. The differences on medial stimulation are usually restricted to a change in the time relations of the two roots. Occasionally a somewhat greater difference occurs. For instance, one medial may not trigger the larger fiber in one or both roots when the other does so. Because of the symmetrical effect of stimulation of one medial, the close resemblance is in this case to be expected.

More interesting is the comparison between medial and lateral fiber stimulation. In many cases the discharges are still amazingly similar, showing the same number of repetitions and differing only in minor respects. In other cases, clear differences do occur, which are fairly constant. They usually consist of a shorter discharge on lateral stimulation than on medial. Figure 4 gives an example of a preparation in which the lateral discharge was considerably different from that of the medial. This figure shows too that it is sometimes difficult to decide how long the discharge really is. On the lateral stimulation, the roots of the third ganglion clearly give two discharges of the smaller fiber as a result of the stimulus, but whether the delayed third one is spontaneous or belongs to the discharge cannot be decided. Spontaneous impulses can also occur at the beginning of a series; hence there is always an uncertainty of at least one impulse. In a preparation which shows such a difference between medial and lateral stimulation, the effect is not limited to the roots of one ganglion; the second roots of other ganglia show a similar difference (fig. 4, 5 M and 5 L). Therefore, the effect cannot be ascribed to a purely local peculiarity in one ganglion. Thus the question can be raised whether repetition might be under the control of a special ganglion, like the suboesophageal or the telson. In this connection experiments were performed in which the part of the cord behind the ganglion of which the roots were used was removed. This procedure had no influence on the discharge. In some special experiments, where repetitive discharges were obtained by stimulating the cord between the telson and the 5th abdominal, the part of

the cord anterior to the ganglion was cut off, without however any effect again. It must therefore be concluded that, if other structures are involved in regulating the length of the discharge, this influence is not quickly removed by isolation.

C. The spontaneous activity in roots of different ganglia. If the second roots of different ganglia are compared with

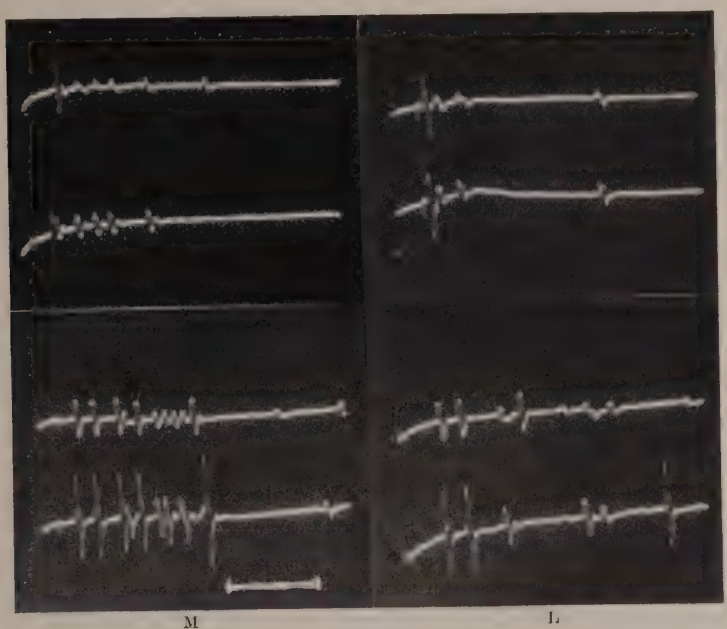


Fig. 4 Comparison of second root discharges of third and 5th abdominal ganglion on stimulation of a lateral and medial giant fiber. Upper two pictures: discharges on right medial, and left lateral stimulation of top: right, bottom: left roots of third ganglion. Lower two pictures: similar discharges in corresponding roots of the 5th ganglion. Time, 25 milliseconds. Note that all 4 discharges on lateral stimulation are shorter than those on medial. Also, that discharge of 5th root shows a different make-up in component fibers.

regard to their spontaneous activity, it is observed that there is no distinct correlation between individual impulses, as is the case in the symmetrical roots of one ganglion. On the other hand, an over-all similarity in the number of impulses per second is usually present. This similarity is especially striking when the spontaneous discharge rate changes, for

within a short time the change then occurs in all the roots of the different ganglia of the cord. The preparation as used often shows periods of "unrest," at which time the stumps of the legs start to move, and the preparation may even actively change its position in the dish, to the extent that the head clamp allows shifting. At such times spontaneous activity increases in all roots. This observation raises the question whether spontaneous impulses arise in the ganglia themselves or are caused by nerve impulses coming from other parts. A series of experiments was performed in which a given ganglion was gradually isolated from the others in front and in back. Often this procedure does not interfere with the activity, and the completely isolated ganglion may for a long period go on to send out impulses. However, there are then no longer any noticeable variations in the activity, which is always of the order of 5 to 10 per second. Changes in activity do sometimes occur after a cut, especially when the rate happens to be high at the time. Lowering of the frequency has been noted as the result of such a cut, and there have also been instances in which an increased rate was encountered. There can be no doubt that, though the spontaneous rate is inherent in each ganglion, there are fibers between the ganglia which have a regulatory influence. These results agree well with those obtained by Prosser ('34) on the over-all spontaneous activity under similar conditions.

D. Comparison of the discharge of different roots of different ganglia. When the discharges of a first and a second root of one ganglion are compared with each other it is found that they are much less similar than are the discharges of the two second roots (fig. 5). In general, the first roots give an even more prolonged discharge than do second roots, and the number of fibers participating is larger. However, the first root discharge starts also often with a larger spike, and comparison between the two first roots of one ganglion shows that their total discharges are very much alike. It is of interest to note that, when lateral and medial giant fiber impulses result in clearly different discharges in the second roots, a

very similar difference is present in the discharges of the first root, as is illustrated in figure 5. In this picture the discharges of the homologous roots of the third and 4th ganglia are even more similar than the two discharges in any one root caused by the stimulation of the two giant fibers.

When third roots are led off, it is found that when they give a repetitive discharge at all, this is limited to a small

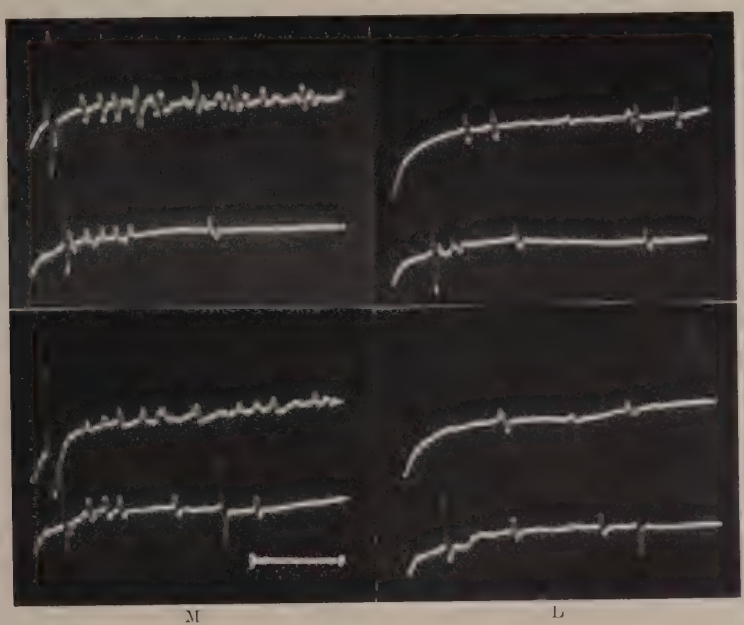


Fig. 5 Comparison between discharges of first and second roots of the same ganglia, both on medial and lateral stimulation. Upper two pictures: medial and lateral stimulation discharges of top: right first root; bottom: right second root of third abdominal ganglion. Lower two pictures, top: right first root; bottom: right second root of 4th abdominal ganglion. Same preparation as figure 4. Time, 25 milliseconds.

fiber, whose potential is very small compared to the potential of the large motor axon, which usually gives a single discharge (cases of double discharges have been previously reported, however, Wiersma, '47). The duration of the repetitive discharge of the small fiber is shorter than that of the second root, and has not been especially studied. However, the evidence is again that as in the case of the first and second roots,

preparations which show a long discharge in the second root will be the ones to give a long discharge also in the third roots.

E. Spontaneous activity and root discharge. It seemed likely, from several considerations, that there would be a close connection between the amount of spontaneous activity and the duration of the root discharge. Thus it might be considered that a fairly high spontaneous rate would represent a state in which the fiber would also be easily brought to repetitive firing. The results do not sustain the existence of such a correlation. In preparations in which a giant fiber gives only very few, for example two, smaller fiber potentials, the rate of spontaneous discharge was not lower than in a preparation in which a great number of repetitions was brought about. Also, when the number of repetitions is reduced by more frequent stimulation, no obvious change occurs in the spontaneous discharge rate. In order to obtain more information on this point and also to investigate factors which influence repetitive discharge, a number of experiments were performed in which the perfusion medium was changed by drugs and in ion content. Of these experiments, those performed with nicotine, acetylcholine, pyrethrum and calcium-poor perfusion fluid will be reported.

Nicotine has, as previously shown (Wiersma and Schallek, '48), a profound action on the synaptic transmission between the giant fiber and the motor fibers of the third root. In low concentrations, it facilitated this transmission, in higher ones it blocks. In the present experiments it was found to have a very similar action on the giant fiber — second root transmission. Thus in low concentrations (10^{-6}) it made the potential of the larger fiber appear, when this potential had been absent before (fig. 6). In some cases, it was even possible to show that the same effect could be obtained for the smaller fiber when it happened not to fire at all. As expected, with stronger concentrations, like 10^{-5}, a block occurred regularly after some time. Nicotine even in the lowest concentration used (10^{-6}) was effective in increasing the spon-

taneous activity in the smaller fiber. Its action was especially useful since more than other drugs it has the tendency to increase the activity in this fiber only. In no instance was any influence discernible which enhanced the length of the repetitive discharge. All types of preparations have been used with weak and also stronger solutions. The preparations in which only a few impulses (two or three) occurred on giant fiber stimulation would seem the most favorable for showing an increase in afterdischarge, but they definitely failed to show any such response (fig. 6). Neither did preparations

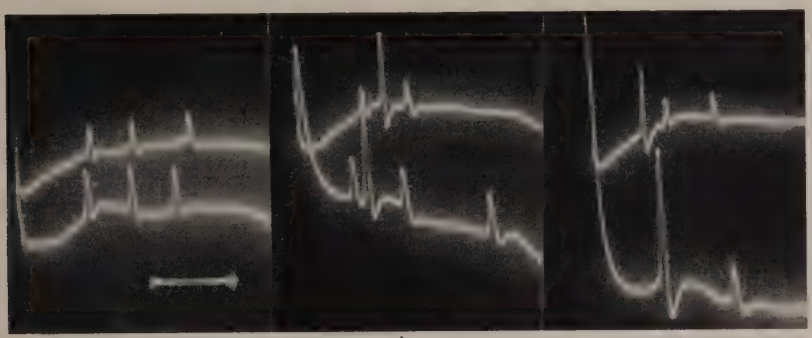


Fig. 6 Influence of nicotine on repetitive discharge of second roots of third abdominal ganglion. Stimulation by right medial giant fiber. Top: right root; bottom: left root. (a.) before nicotine. (b.) 4 minutes after establishing a concentration of 10^{-6} nicotine in the bath. Notice the appearance of the fast fiber by the facilitating action, but the absence of any increase in numbers of repetitions. (c.) 7 minutes after nicotine addition. Further facilitation, accompanied by reduction of repetitive discharge. Complete block occurred after 15 minutes. Time, 10 milliseconds.

in which a long repetitive discharge was obtained show any such effect. Whereas nicotine in a facilitating concentration was found to protect the direct transmission in the third root from fatigue, it does not seem to protect the afterdischarge at all from shortening with repetition. The only effect on the afterdischarge which is obtained is that with higher doses it decreases in length. The reason for this is not quite certain; while it may be partially an effect of the drug on the transmission process at the synapse before blocking becomes complete, it is almost certainly also due

to a decrease in excitability of the postsynaptic fibers caused by the high rate of "spontaneous" activity (40 to 50 per second), which these concentrations of nicotine cause. Even after a complete block is established between the giant fiber and the root potentials, the spontaneous activity is still present, though it tends to decrease in frequency. In isolated ganglia, nicotine was found to have an influence on the spontaneous firing similar to the effect when the whole cord is present.

Acetylcholine failed to have any effect on the duration of the discharge, even in high concentration. Thus a final solution of 5×10^{-5} acetylcholine did not in any way affect the discharge of a preparation which showed one fast and two slow spikes in the second roots of the third ganglion. The influence on spontaneous activity was also not pronounced, a slight increase being obtained with high concentrations of the drug (cf. Prosser, '40b).

Pyrethrum gives a very high degree of spontaneous activity which in contrast to that of nicotine occurs in a great number of fibers. These doubtless include sensory fibers. The activity is often in bursts, with quiet periods in between. During bursts, the repetitive discharge is either cut short or, more often, is completely unrecognizable. During quiet periods it appears again, and then is sometimes completely like the discharge before the administration of pyrethrum or, more often, is somewhat shortened, presumably because of the stimulation of the fiber during the activity periods. In no case was there any evidence of a lengthening of the after-discharge, nor could it be demonstrated that stimulation of the giant fiber with single shock brought about a general period of activity.

Ca-free or Ca-poor (25%) perfusion solution also enhances spontaneous activity, but especially in the smaller fiber, like nicotine. This is in contrast to results obtained by Prosser ('40a) in which Ca had little effect on the total amount of spontaneous activity of the cord. In no case was a lengthening of the repetitive discharge observed. However, Ca-poor solu-

tions have a marked influence on the excitability of the giant fibers themselves. The lateral giants are especially prone to give repetitive discharges under these circumstances. Unless the origin of the resulting root fiber potentials is understood, one may mistake this for a lengthened repetitive discharge, though it is caused by the giant fibers, which then usually cause a single motor discharge in the second root for each giant fiber impulse.

These last findings correspond with preliminary experiments in which two impulses were made to follow each other quickly in a given giant fiber. No special study has been made for this type of situation, but it is clear that this method is not effective in obtaining long-lasting afterdischarges from preparations that do not show them on single impulses.

DISCUSSION

Since crustacean nerve fibers have a strong tendency to respond with repetitive impulses, it is not too surprising to find a central mechanism which, as it were, takes advantage of this property. It is also known that peripheral fibers differ in the readiness with which such repetition arises. The view taken here is that the repetitions observed after giant fiber stimulation in the post-ganglionic fibers are comparable to the repetitions of the peripheral fibers and do not depend on reexcitation of the post-ganglionic fibers by circuits with different delays. The main argument for this proposition is the high frequency rate of discharge which is present in most cases and which must be near the refractory period of the element. Even peripheral axons cannot be driven at frequencies higher than those obtained in the afterdischarge, except for the first few impulses. The structure of the abdominal ganglia would also seem to be in favor of direct excitation, because its relative smallness and dearth of elements would militate against the existence of the complex reentering circuits which it would be necessary to postulate were the observed phenomena to be explained on the basis of reexcitation. Another factor which seems to favor direct influence

by the primary preganglionic impulse is the lack of correlation between the "spontaneous" impulses and the length of the afterdischarge. If spontaneous impulses arise in the fiber, because of firing of another element in the ganglion, it would be likely that "reverberation" would be much more effective during high activity than low activity. The fact that this does not occur means that the same pathways are not involved in the two phenomena.

That phenomena comparable to afterdischarge can be brought about without the existence of reverberating circuits has been shown directly in the case of the peripheral sympathetic ganglia by Larrabee and Bronk ('47). In peripheral fibers one impulse in a fiber may set up a row of discharges at a focal place, which can be obtained by drugs, slight damage or electrotonus (see Bullock and Turner, '50, for giant fiber of earthworm). Bremer and Bonnet ('49) discuss the possibility that afterdischarge for motor axons in the spinal cord of the frog is due not to "reentry" circuits but to catelectrotonus. From this they conclude that there should be a relation between spontaneous activity level and afterdischarge.

With regard to the genesis of the spontaneous impulses in the second roots, it is quite possible and even likely that the post-ganglionic element may at times be solely responsible for its own "spontaneous" rhythm. The correlation between the two roots of one side show, however, that this activity can be transmitted to a certain degree to other elements. The transmission of impulses between the two smaller motor fibers in the second roots is best, but not necessarily, pictured as synaptic; and the same synapse might serve to let the two smaller fibers fire synchronously in the repetitive discharge. The repetitive discharge, on the other hand, may also be brought about by direct synapses of both root fibers with three of the giant fibers, as occurs in the case of the giant root fibers of the third roots. The results do not yet make it possible to distinguish between these explanations. For the large fiber of the second root, the indications are that the

latter explanation is correct, since in some preparations the large fiber on the *heterolateral* side fires only. For the smaller fibers, however, the stimulated side has almost in all cases clearly the lead, which would be compatible with the first explanation. This explanation would have the advantage of a more compulsory correspondence between the two sides than would be offered by the other arrangement. In this respect, it is of significance that the correlation between the number and timing of impulses of the large action potentials is always so much less pronounced than that in the case of the smaller ones. If a synapse between the root fibers exists its transmission should then be in a one-to-one fashion. Experiments in which the second root was stimulated on one side, the other side led off tended to confirm these conclusions; the large fiber did not respond and the smaller one could be driven. Since sensory fibers are also stimulated this fact does not allow to conclude that a synaptic transfer between the two smaller fibers does exist, only that it may be present.

One might be inclined to ascribe the difference in the discharges of the different roots to difference in the viability of the ganglia. This would mean that the more posterior ones would be in better condition than the anterior ones with regard to the first and second root repetitions. Against this view pleads the fact that the transfer of the giant fiber potential to the third roots in the anterior ganglia is more fatigue-resistant than in the posterior ones, a fact that was mentioned in previous publications and has been confirmed in the present investigation. Therefore, it seems likely that even in the normal animal there are longer discharges in the first and second roots of the posterior ganglia and if this supposition is true it would mean that the posterior segments would show a proportionally greater contraction of the extensor musculature. This difference might well have a functional significance in the swimming movement.

The symmetrical discharges of the roots are obviously of importance in order to secure symmetrical movements of

the tail musculature. The most difficult to understand is how the symmetry is brought about on stimulation of a lateral fiber, especially when the repetitive discharge obtained differs from that on medial giant fiber stimulation. If, as explained above, the smaller fibers are connected by a synapse, its functioning might explain the symmetry, were it not that the fiber in the heterolateral root is also stimulated by its own homolateral giant fiber. This stimulation will arrive later under experimental conditions, but should have an influence. It is imaginable that such influence might be inhibitory in character and that the difference between the effects of lateral and medial stimulation may be due to this factor. At any rate the conditions must be rather complicated, and no satisfactory explanation is as yet possible with the evidence at hand.

Perhaps the most unexpected finding in the present research has been the absence of a correlation between the tendencies to afterdischarge and to spontaneous activity. The normal as well as the drugged preparations all tend to show that the two are independent of each other, within wide limits at least. This gives rise to the necessity of locating their genesis at different points in the post-ganglionic fiber, and makes it more difficult to consider a state of catelectrotonus as responsible for either, since such states would be expected to spread rather easily from one part of the element to another and to have a considerable influence upon each other.

If the thesis that the afterdischarge is due to a simple synaptic transmission process is accepted, it becomes interesting to compare the effects of the synapses with different properties on the relation between in- and output of the crayfish central nervous system. Prosser ('35) has shown that at least certain synapses on the input side are of the kind in which temporal and spatial summation are necessary for transmission. In particular, his experiments concerned fibers from hairs on the telson. By this mechanism reactions of the animal to minor disturbances in a few of these exteroreceptors are prevented, and reactions take place only after

a number of impulses have reached the CNS. Within the CNS itself this method is used for certain reactions, but not for others; in many cases, single intercentral fibers require repetitive stimulation before reactions take place, an indication of the necessity for facilitation. However, in other cases, a single impulse is sufficient to cause a single impulse in the output as the synapses involved are one-to-one. This relationship is well known from the giant fiber impulse effect on the flexion of the tail musculature. However, it is only because "fast" nerve-muscle systems are involved that this "information" does not after all become lost again in the periphery. If a similar single impulse were to be set up in a slow system or for that matter, in a fast system which does not respond to a single impulse, it would still become lost. One may see an impulse in a giant fiber as a very important event; once it is achieved, it should be responded to. The present investigation shows another way in which this result can be obtained that does not depend on peripheral systems which react to single motor impulses. These motor fibers are involved in tail extensions, but their peripheral course will have to be traced, in order to see whether they go to the true extensor musculature or take part in the innervation of the "muscle receptor organs" of Alexandrowicz ('51). In any case the number and frequency of impulses found in a long discharge will certainly result in a noticeable contraction, even in the case of a slow fiber. If the stretch musculature itself is involved, one might well picture the effect as helping in relaxation and readying the tail for a subsequent flap by a future giant fiber impulse.

SUMMARY

Single impulses in the intracentral giant fibers of the crayfish, *Cambarus clarkii Girard*, regularly cause a repetitive discharge in the roots of the abdominal ganglia.

These discharges, especially those of the second roots, have been studied under various experimental conditions.

They occur especially in one fiber, at least in the case of anterior ganglia. Another, larger fiber, usually responds in these ganglia with a single impulse, but may also show repetition.

The same fiber, which shows repetition, is normally spontaneously active, usually at a rate between 5 and 10 per second.

The frequency of the repetitive impulses is high and may be above 300 per second. The number of impulses in each afterdischarge is variable, but is seldom more than 10.

There is no connection between the number of discharges on giant fiber stimulation and the frequency of the spontaneous discharge.

With repetition of stimulation even at a low rate of the giant fiber, the number of impulses in the afterdischarge diminished and may be reduced to one impulse long before the synapse is completely blocked by fatigue.

The afterdischarge of the two symmetrical roots of a ganglion on stimulation of one giant fiber is usually almost identical.

There is often a very close similarity in the discharges of a given root on stimulation of different giant fibers, though in some preparations, the results may show considerable differences.

Similarly, comparison of the discharges of second roots of different ganglia for the same or different giant fiber impulses shows considerable similarities in some preparations, but not in others.

The duration of an afterdischarge is more a function of the preparation than of a given root, unless the latter is damaged.

Agents like nicotine and Ca-free solutions, which bring about large increases in spontaneous activity, are without effect in prolonging the afterdischarges. When the spontaneous activity becomes too great, the afterdischarge becomes shortened.

After the synapse has been blocked by appropriate nicotine solutions, the spontaneous activity may still be high.

Reasons for considering the afterdischarge as caused by a single synaptic junction, which delivers repetitive impulses, are discussed.

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THE CHOLINESTERASE OF THE MOTOR END-PLATE REGION

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SIX FIGURES

Recently a method for the histochemical localization of cholinesterase was introduced by Koelle ('50) in which it was demonstrated that discrete black aggregations of precipitated copper sulphide could be obtained, as a result of cholinesterase activity. In striated muscle, as is well known, cholinesterase is confined exclusively to the motor end-plate region and this method therefore serves to stain these regions selectively.

Owing, however, to the high cholinesterase activity of the end-plate region the quality of the staining that can be achieved in fresh material, for example the diaphragm, is exceedingly poor (fig. 1) and it cannot be assumed that the black irregular precipitates bear any resemblance to the structural details of the motor end-plate. Qualitative interpretation of the results is therefore rendered very complex.

To overcome this difficulty, the diaphragm was, on removal from the animal, fixed in 10% formol-saline (i.e., 4% effective concentration formalin) for 48 hours at room temperature before proceeding with the rest of the method. The histological appearance was thereby strikingly altered and although the activity of the enzyme had, as judged indirectly, been reduced, the localization now was clear and precise (figs. 2, 3 and 4).

It must be emphasized that not only had the appearance of the cholinesterase containing motor end-plates been altered but, as was to be expected, that of the rest of the muscle

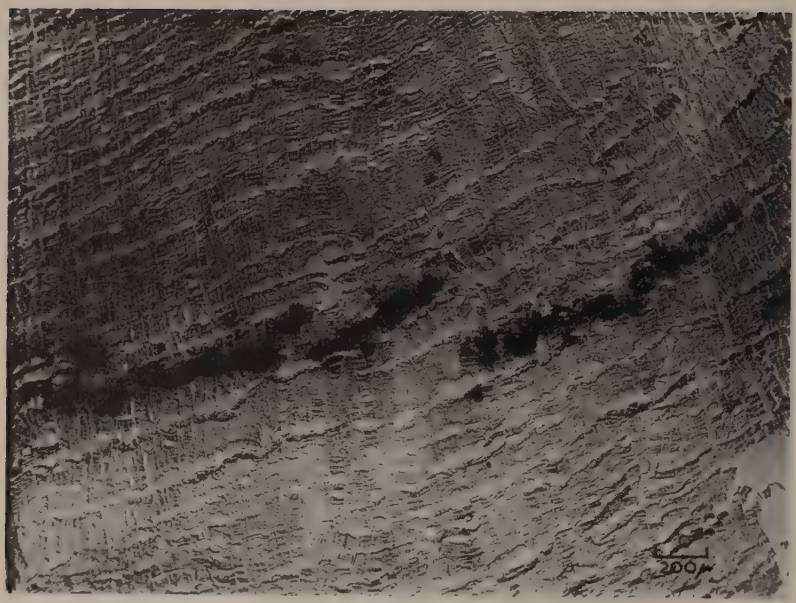

Fig. 1 Rat diaphragm. × 50.

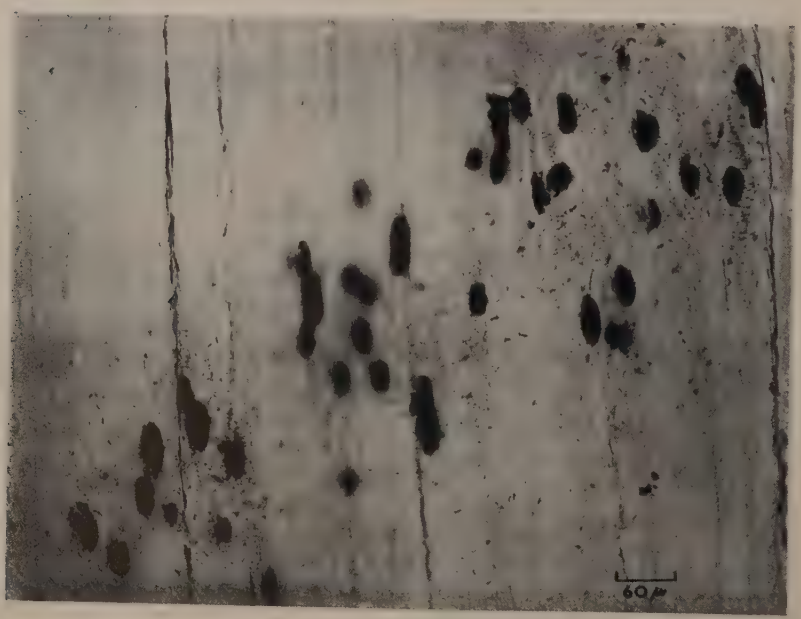

Fig. 2 Cat diaphragm. × 170.

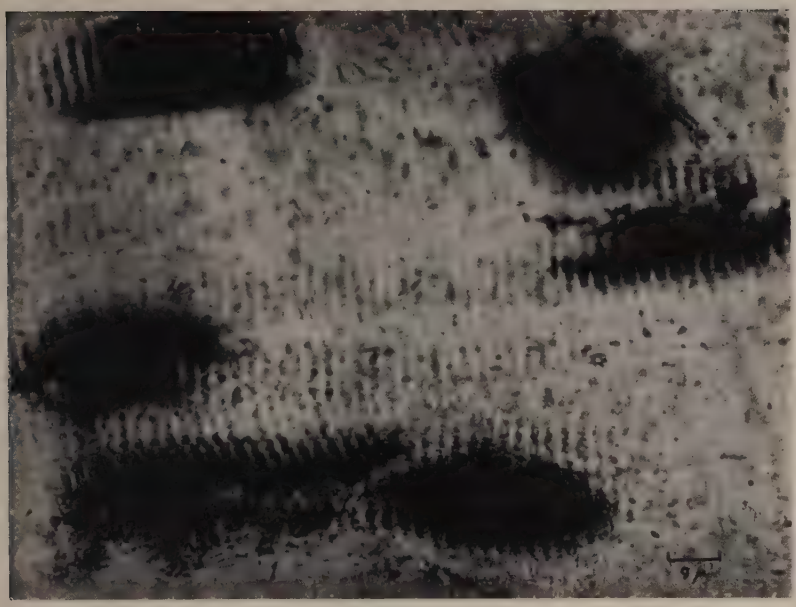

Fig. 3 Cat diaphragm. × 1,100.

Fig. 4 Cat diaphragm. × 1,100.

fiber as well. The cross-striations which could not be distinguished before could now be seen and it was evident that the greater part of the cholinesterase was localized on the surface of the anisotropic bands immediately below the motor end-plates. The terminal portions of nerve fibers cut either in transverse or longitudinal section were also heavily stained. These are known to contain the enzyme in high concentration.

Fixation before using a histochemical procedure designed to locate cell components, introduces a number of obvious difficulties so that the thereby altered appearance may be due to fixation artefacts cannot be ruled out. On the other hand, the distortion in unfixed tissue in itself prevents accurate localization, especially in striated muscle where retraction clots may seriously alter the histological appearance and the motor end-plates are, in such preparations, barely recognizable.

Critical tests have shown that the histological picture presented in figures 2, 3 and 4 cannot be ascribed to a false positive reaction, special adsorptive qualities of the areas stained, or diffusion of the reaction products. Thus, incubation in a substrate containing all reagents except acetylthiocholine produces no staining; similarly when the acetylthiocholine is replaced by butyrylthiocholine no staining is evident after 30 minutes. Incubation with 10^{-3}M DFP for 30 minutes before transferring the sections to the substrate likewise has the effect of abolishing all staining. It is therefore reasonable to assume that no false positive reaction is obtained.

That diffusion does not appear to be a factor in the staining emerges from the fact that the average size of the stained end-plate region is the same irrespective of whether the incubation time is 5 minutes or two hours though of course the depth of staining in these two extremes is very different.

A special adsorptive capacity of the motor end-plate region for copper sulphide (the end products of the entire chain of reactions in this method) can also be neglected since placing the sections in a substrate containing all reagents except

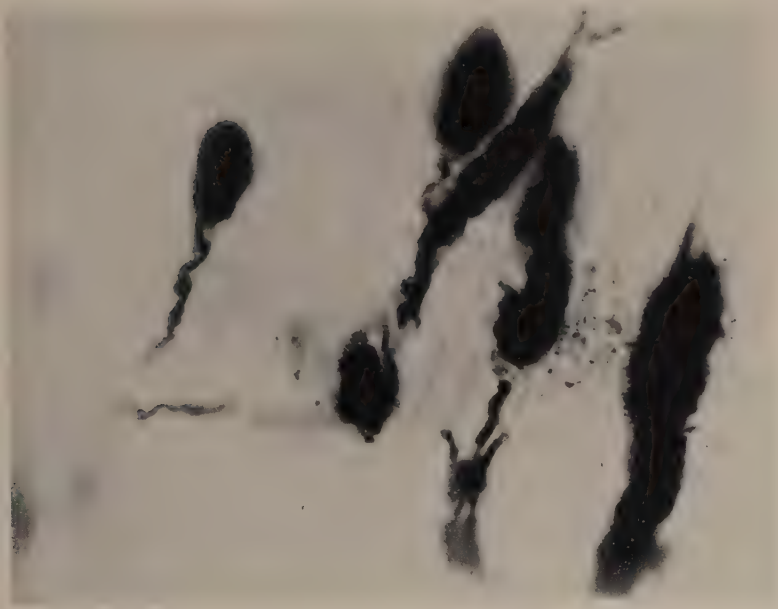

Fig. 5 Cat diaphragm counterstained silver. × 600.

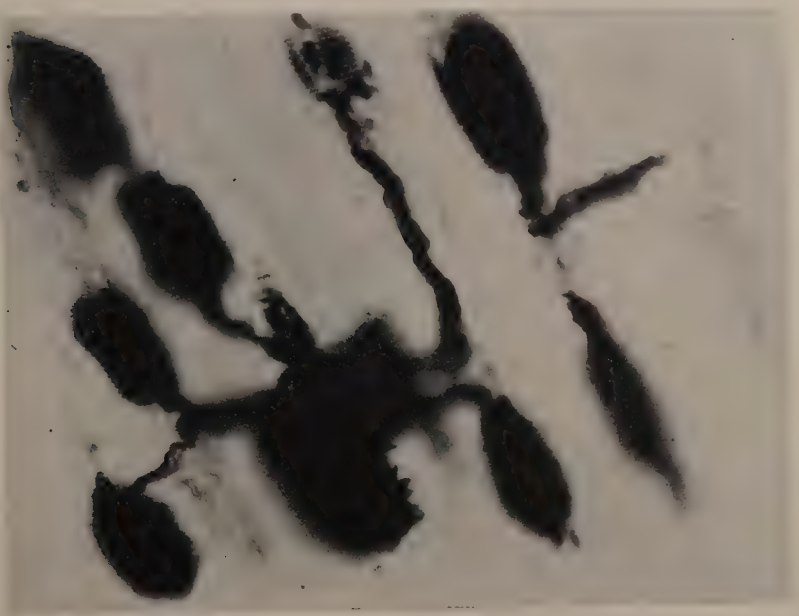

Fig. 6 Cat diaphragm counterstained silver. × 600.

acetylthiocholine, but saturated with copper sulphide produces no staining.

Finally, in order to ascertain beyond doubt whether the stained areas were indeed motor end-plates, sections were counterstained with silver (figs. 5 and 6). It may be seen that they conformed to this criterion.

ACKNOWLEDGMENTS

For valuable suggestions I am very much indebted to my colleague, Dr. R. Barer, and to Mr. E. H. Leach of the Physiology Department of this University.

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DIVISION DELAY BY RADIATION AND NITROGEN MUSTARD IN PARAMECIUM [1]

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NINE FIGURES

One of the major biological effects of radiation is retardation of cell division. In recent years, this phenomenon has been reviewed by Giese ('47), Lea ('47), Luther ('48), Carlson (in press), and Kimball (in press). Much of the previous work has concerned only the first division after irradiation. However, there is some evidence for effects upon later divisions. In particular, the work of Giese ('39, '45) and Giese and Reed ('40) on *Paramecium* exposed to ultraviolet has shown marked effects upon several successive division intervals.

The present paper demonstrates that there is a distinct pattern to these effects on later divisions and that the major features of this pattern are common to division delay produced by ultraviolet and X rays and probably to that produced by nitrogen mustard. On the basis of this pattern, two major parts to the phenomenon are recognizable, (1) an effect upon the first division and (2) a delayed effect which retards mainly the 3rd, 4th, and 5th divisions. The paper gives descriptions of this pattern, develops certain quantitative methods for routine as well as detailed investigations, and offers certain hypotheses to account for the phenomenon.

The experiments with ultraviolet and X rays were carried out by two of us, Dr. Geckler joining with us in the nitrogen mustard experiments.

[1] This work performed under contract W-7405-eng-26 for the Atomic Energy Commission.

MATERIALS AND METHODS

Stocks of paramecium. All the work with ultraviolet and X rays and part with nitrogen mustard was done with stock delay to the various division intervals, but the total effect was done with a stock of *P. caudatum* collected some years ago by the senior author.

Culture. The paramecia were kept in pyrex triple-depression spot plates using the standard lettuce infusion, *Aerobacter* medium (Sonneborn, '50). Since clones of *P. aurelia* cannot be maintained for long periods of time because autogamy occurs spontaneously every two to three weeks, a new clone was obtained once a week by autogamy and maintained as a daily isolation line during the next week. Thus, all animals for any one experiment were from a single clone, but different experiments were usually performed with different clones. However, the different clones were all descendants by uniparental reproduction from a single homozygous ancestor and so could differ genically only as a result of spontaneous mutation. On the day before an experiment, the clone was expanded into a series of isolation lines from which dividing animals were collected over a 15-minute interval and exposed within two hours after collection. Thus all animals for a particular experiment were at about the same stage in the division cycle when exposed, and animals were used in approximately the same stage for all experiments. *P. caudatum* is not known to undergo autogamy, and the two experiments with it used vegetative descendants of a single ancestor.

Ultraviolet irradiation. In some experiments, a 15-watt General Electric germicidal lamp was the source while in others a large quartz monochromator with a medium pressure mercury arc was used. The methods employed with the latter are given by Kimball and Gaither ('51). With the germicidal lamp, the animals were suspended in culture fluid diluted about 1:1 with 10% Ringer's solution. A layer of this suspension approximately 2 mm deep was placed in an uncovered glass petri dish, 5 cm in diameter, and this dish was

rotated slowly on a turntable 60 cm from the lamp. Measurements were made of the germicidal lamp with a General Electric germicidal ultraviolet intensity meter and of the monochromatic source with a thermopile and sensitive galvanometer. The dose rate varied from 0.8 to 5 ergs/mm²/sec. for the different sources and wavelengths used. Within a few minutes after irradiation, the paramecia were removed from the vessel with as little fluid as possible and pipetted one by one into depressions of culture fluid.

X irradiation. A General Electric Maxitron 250 machine operated at 250 kvp and 30 ma was used. No filtration was added to the inherent filtration of 1 mm of aluminum. The beam was directed upward through the mica bottom of a dish formed by cementing a sheet of mica 23 μ thick to a lucite ring 5.5 cm in diameter and 2.5 cm high. The dish was filled to a depth of 1.5 cm with a light grade white paraffin oil, and 30 to 50 paramecia were placed in a small drop of culture fluid on the surface of the mica.

The problem of measuring the highly heterogeneous beam of X rays from the unfiltered source has not been adequately solved. As a working procedure for standardization, a 250 r nylon Victoreen chamber was used. Routine monitoring was done in air at 49 cm from the target, and the results were extrapolated to 7.7 cm (the position of the animals) on the basis of previously made measurements of the relation of intensity to distance from the target. The dosage rate calculated in this fashion was approximately 68,000 r/minute. As soon after irradiation as possible, the paramecia were removed from the small drop to about 0.5 cm³ of fresh culture fluid and then isolated individually into depressions of culture fluid.

Nitrogen mustard exposures. The animals were exposed to freshly prepared solutions of methyl-bis (ββ′-dichloroethyl) amine hydrochloride in 0.005 *M* phosphate buffer adjusted to pH 6. The exposures were made at room temperature (25°–30°C.) by adding the animals, concentrated into a very small volume, to approximately 0.5 cm³ of the mustard solution in

a depression slide. When the exposure was completed, the solution was diluted to approximately one-quarter its original concentration with culture fluid, and the paramecia were removed as rapidly as possible by a capillary pipette to fresh culture fluid. As little of the mustard as possible was transferred with the animals. They were passed through two more changes of culture fluid, and isolated individually into depressions. This method is essentially that used by Geckler ('50) in his investigation of the genetic changes induced by nitrogen mustard in *Paramecium.*

Observations. In most experiments, 30 animals were isolated for each treatment group. The observations were made at 1-, 6-, or 24-hour intervals. At each observation, the number of animals was recorded separately for each line of descent. Once a day, one animal from each depression was transferred to a depression containing fresh culture fluid, and the remainder were discarded. In this way the animals were maintained in the log phase of growth without appreciable crowding and the numbers remained small enough to be readily counted.

Calculations. The original data consisted of the numbers of animals present at given times. It was desired to convert these data into time between successive divisions. A set of somewhat arbitrary procedures was adopted for this purpose.

For the hourly observations, the time of division was taken as the mid-point between the last observation at which no animals in the depression had undergone division and the first at which all had divided. The same convention was adopted for 6-hourly observations, with two exceptions. A line of descent was considered to have divided at the time of observation if one or more animals were observed in the process of division or, if it was obvious from the size relations and numbers, that only part of the animals had divided. One division was assumed to have occurred at the beginning and one at the end of a 6-hour interval if two divisions occurred between observations, since 6 hours was nearly the division interval for the controls.

A somewhat different set of conventions had to be adopted for the daily observations since several divisions frequently occurred in one day. It was assumed that the divisions on a given day occurred at equal intervals and that the last division occurred at the end of the 24-hour period. When one or more days passed without division, these days were added to the time for the interval in question. Such a procedure is, obviously, somewhat arbitrary; but it allows some estimate of the effect by division interval rather than by days. It also has the advantage that unlike other procedures, it yields equal intervals when the number of divisions per day remains constant. In a later section, the results obtained by this method will be compared with those from more frequent observations.

In the paper of Kimball and Gaither ('51) and in some of the data in the present paper, the arithmetic mean of the time between certain divisions was used. However, the reciprocal of the time has a more nearly normal distribution and the standard error of its mean is only slightly, if at all, a function of the mean. Therefore, it is a more satisfactory form for the data since statistical procedures can be used with greater assurance. The reciprocal times can be expressed as divisions per hour by measuring time in hours and multiplying the number of division intervals which are included. This quantity is the one which will be used in this paper.

We are indebted to various members of the Mathematics Panel of the Oak Ridge National Laboratory for discussion of the problems involved in measuring division delay and carrying out some of the statistical procedures. However, responsibility for the final form in which the calculations were made rests upon us.

RESULTS

Ultraviolet

The major features of delay in cell division by ultraviolet have already been given briefly by Kimball and Gaither ('51).

The first division interval is markedly longer than normal while the second interval, though longer than normal, is shorter than the first. However, at higher doses, either the 3rd or 4th interval is often very long indeed. Sometimes a week or more elapses before the paramecia divide again. The 5th and 6th intervals are usually longer than normal but progressively shorter than the preceding intervals; the normal rate is restored by the 6th to 8th division.

An experiment with frequent observations. Thirty animals were isolated from an unirradiated control group and from groups given 500, 1000, 1500, 2000, and 2500 ergs/mm^2 of monochromatic 2650 Å ultraviolet. At the highest dose, 5 lines of descent failed to survive; but otherwise all survived and recovered the normal rate of division. Hourly observations were made for three days after irradiation. During this time, all lines of the controls and the 500 ergs/mm^2 group completed at least 6 divisions and all other dose groups completed at least two divisions. Observations were made every 6 hours for 5 days more during which time all lines of the 1000 and 1500 ergs/mm^2 groups and 19 lines of the 2000 ergs/mm^2 group completed the 6th division. Daily observations were continued until all viable lines completed at least 12 divisions.

The rates, as divisions per hour, and their standard errors are plotted for successive division intervals in figure 1. The scale on the abscissa in figure 1 and also in figures 7 and 9 is obviously discontinuous, and so the lines connecting the points are for convenience and do not imply a continuous change in rates. There is a marked effect upon the first interval, somewhat less upon the second, and again a marked effect upon the 3rd through the 5th intervals. Recovery is nearly complete by the 6th division so that the 7th interval is of nearly normal duration. This pattern is nearly constant over the dose range used and suggests that there are two major parts to the delay, an effect upon the first interval and a delayed effect upon the 3rd, 4th, and 5th.

The control intervals show significant variations which appear to be related to the daily transfers. However, passage through a given interval occurred at quite different times in the control and irradiated groups. Thus, a direct correction for control variation cannot be made, even if it

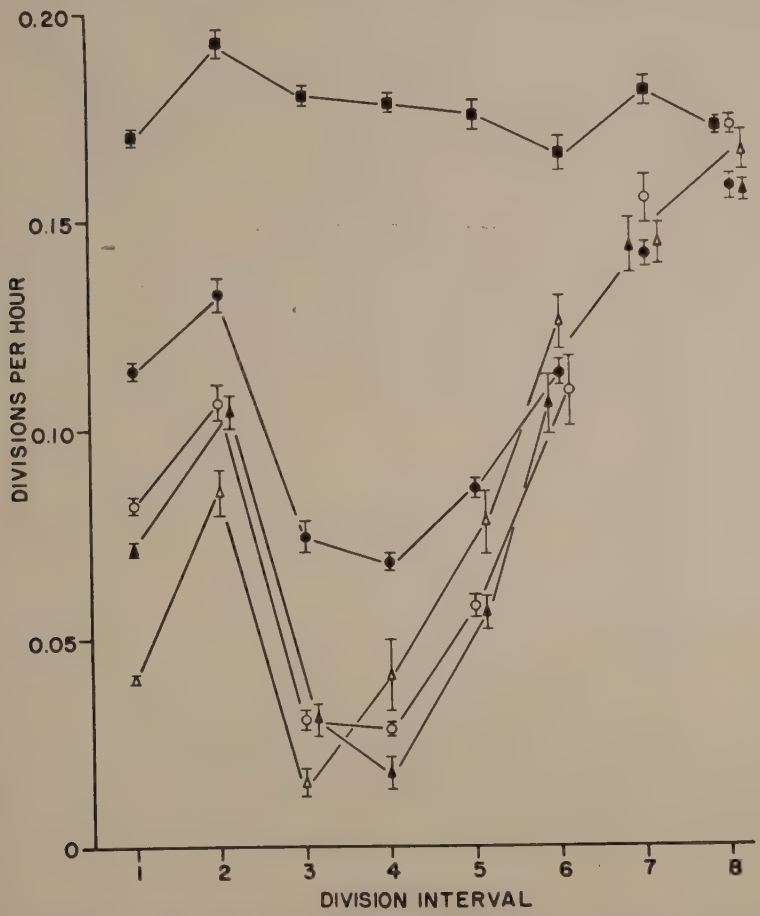

Fig. 1 Divisions per hour and standard errors for successive division intervals after irradiation with monochromatic 2650 Å ultraviolet. The first division interval is that between irradiation and the first division; the second, that between the first and second divisions, etc.

■ — none
● — 500 ergs/mm²
○ — 1000 ergs/mm²
▲ — 2000 ergs/mm²
△ — 2500 ergs/mm²

be assumed that the factors influencing the controls would act in the same way upon the treated animals.

Further examination of individual lines of descent reveals that, at higher doses, there is a marked negative correlation between the duration of the 3rd and 4th intervals, as shown in figure 2. At low doses, this correlation does not exist; but,

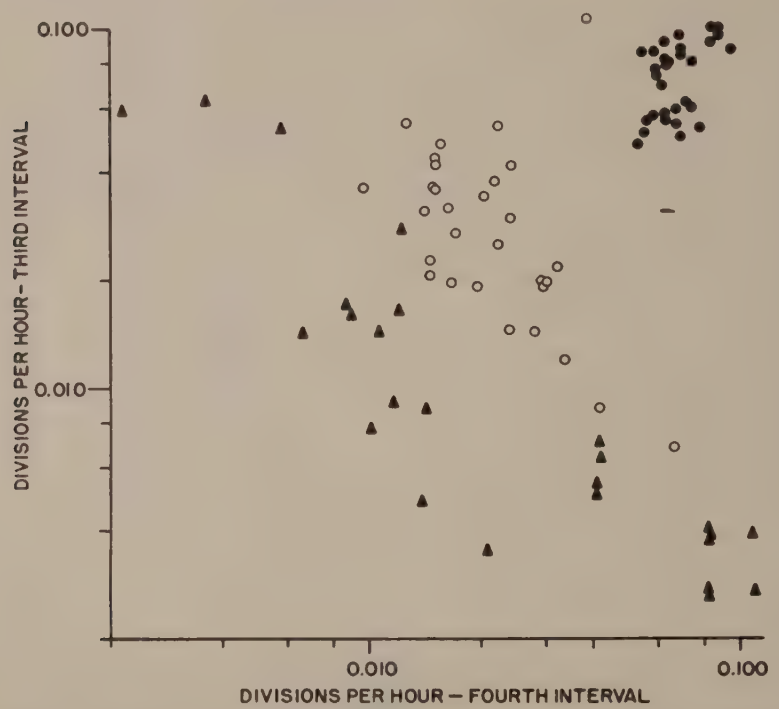

Fig. 2 Divisions per hour for the third interval plotted against divisions per hour for the 4th interval for the same line of descent. A log scale was used to spread the points at the higher doses.

● — 500 ergs/mm² ▲ — 2500 ergs/mm² ○ — 1500 ergs/mm²

at higher doses, if one interval is very long, the other is relatively short. If one is of medium duration, the other is also medium. In the present experiment, no correlation was found between other pairs of intervals. However, in some other experiments, a few lines with very long 1st, 2nd, 5th, or 6th intervals have been found; and, in these instances, all

other intervals were rather short. Thus in many, though not all, lines of descent there is one interval which is very long, but no more than one such interval occurs in any one line of descent. In some lines, even at high doses, no very long intervals occur; but in this case two or more intervals are of intermediate duration.

The longest interval found in a line of descent which eventually recovered was three weeks. Two-week intervals have been relatively common. During such long cessations of division, the animals become small and thin and move quite sluggishly. It is obvious from the fact that they become progressively smaller that they are not synthesizing enough material to keep pace with their needs. Death of such small animals is not uncommon, and the impression is obtained that all these animals are near death so that minor variations determine survival. Those that survive recover the normal rate of division within one to two days of the time at which they first divide again.

Dosage relations for successive division intervals are shown in figure 3 in which the log division per hour is plotted against dose. The form of the curves is complex and the points for the later intervals at the higher doses are not too well established, since they are based upon daily observations. However, the curves for the first and second divisions are based entirely upon hourly observations, and so the sigmoid form of these curves is rather well established. As will be seen, the dose curve for the first division following X rays is also sigmoid. It would be possible to analyze such curves into a combination of two or more simpler relations but, in the absence of any adequate theoretical basis, such an analysis would not be justified.

On the whole, there is relatively little change with dose in the distribution of delay to the various intervals. The minor changes which are brought out by figures 1 and 3 are an increase in the delays in the 3rd and 4th intervals relative to that in the first and, possibly, a shift toward a greater effect upon the earlier intervals. This latter shift also ap-

pears to be shown by the X-ray data which will be given in a later section.

The details of the distribution of the delay to various intervals allow certain measures to be selected as best describing the effect. The best single measure of the total effect would appear to be the reciprocal of the time from treatment to the 6th or, perhaps, the 7th division. The former has been chosen for this purpose. When it is desired to examine the

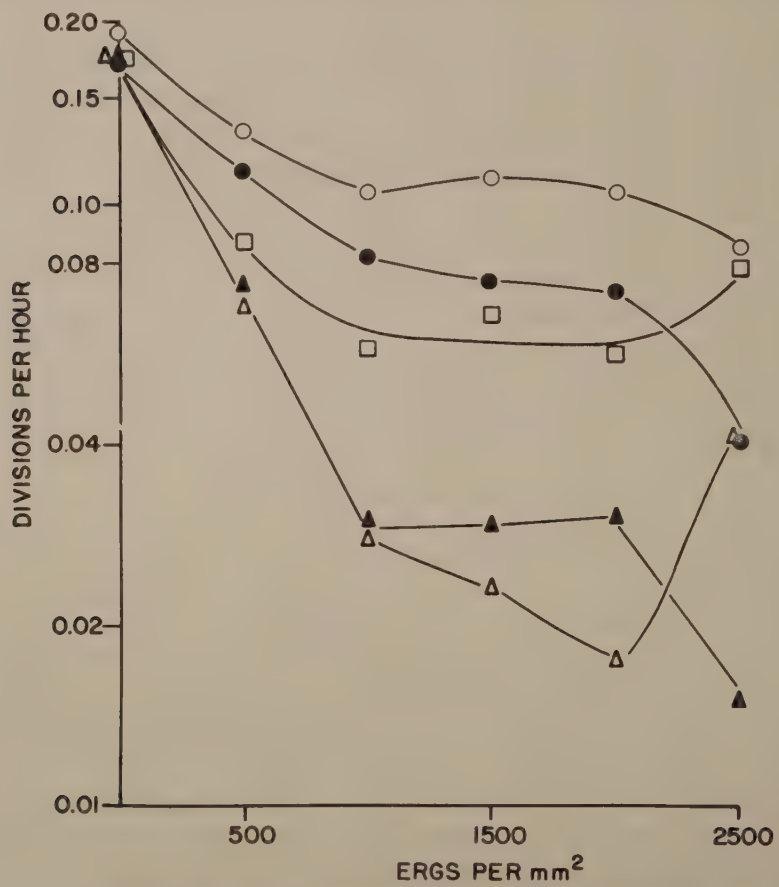

Fig. 3 Divisions per hour for given division intervals plotted on a log scale against dose of 2650 Å ultraviolet.

● — 1st interval ▲ — 3rd interval □ — 5th interval
○ — 2nd interval △ — 4th interval

distribution of delay to the various intervals, the time to the 6th division can be broken down into three intervals; irradiation to the second division (early period), the 2nd to the 4th division (late period), and the 4th to the 6th division (recovery period). For comparison between material with different control rates, it is more convenient to express the reciprocal times as percentages of the control. Data for time to the 6th division expressed in this way are plotted in figure 4. The ultraviolet curve is a composite of two curves: one for material irradiated with the germicidal lamp and observed daily and one for the frequent observation experiment just discussed. There was no reason to suppose that equal doses as measured in ergs/mm^2 incident upon the container would give equal effects for the two kinds of radiation since the methods of exposure and the wave lengths were different. Therefore, the dose scales were chosen to make the 50% points for the two kinds of radiation coincide. Over a rather wide range of doses, the resulting curve can be treated as a simple exponential without too great a loss in accuracy, though it obviously departs from the simple exponential form.

Daily observations. Observations at hourly or even 6-hourly intervals are too time consuming for routine use in measuring the effect of radiation under various conditions. Daily observations are quite feasible, and can be used to obtain estimates of the time to the 6th division, and even of the time between successive divisions using the calculation procedure given in the section on Methods. The question is how nearly do these estimates agree with those made from more frequent observations.

The frequent-observation experiment with ultraviolet was carried out in such a way that it was possible to construct the data which would have been obtained had only daily observations been made and compare the calculations from the constructed with those from the original data. For this purpose, only the intervals which were completed during the

hourly or 6-hourly observation periods were used. The comparisons are given in table 1.

It is clear that errors of some magnitude may be introduced by use of daily observations, especially at low doses.

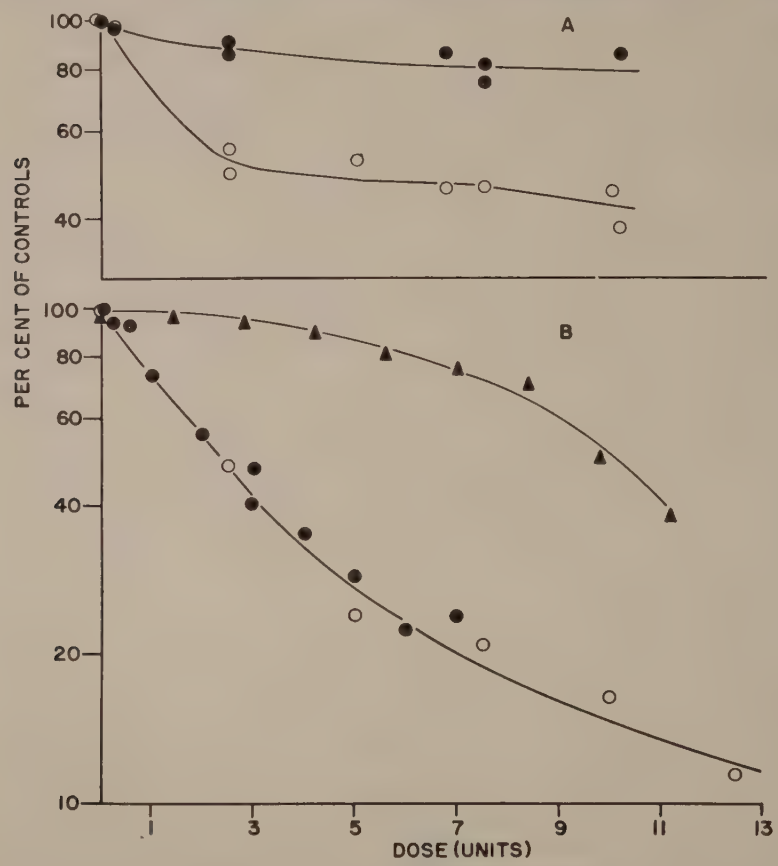

Fig. 4 Mean reciprocal time to the 6th division expressed as a per cent of the control and plotted on a log scale against dose. The dose scales were arbitrarily chosen to spread the data across the graph except that the scales for germicidal and monochromatic 2650 Å ultraviolet were chosen to make the 50% points coincide. One dose unit equals 200 ergs/mm² of 2650 Å ultraviolet, 1000 ergs/mm² of ultraviolet from the germicidal lamp, 25 kr of X rays, and 0.2 mg/ml of nitrogen mustard for 10 minutes.

(A) Mustard
● — *P. aurelia*
○ — *P. caudatum*

(B)
● — Ultraviolet germicidal
○ — Ultraviolet 2650 Å
▲ — X rays

It is also clear that calculations for a single division interval are not very reliable, even at higher doses. Calculations for successive two-division intervals are much better, though even in this case, errors of 30% or more may be anticipated. Calculations for the total period to the 6th division can be made within a few per cent even at the lowest dose used.

TABLE 1

Comparison between calculations of rates based upon hourly and 6-hourly observations and those based upon daily observations

INTERVAL	ITEM [1]	DOSE IN ERGS/MM^2				
		500	1000	1500	2000	2500
0–1	H	0.114	0.082	0.075	0.071	0.040
	D	0.105	0.085	0.083	0.080	0.041
	P	7.9	3.7	10.7	12.7	2.5
1–2	H	0.133	0.107	0.122	0.104	0.085
	D	0.105	0.085	0.083	0.080	0.050
	P	21.1	20.6	25.9	23.1	41.2
2–3	H	0.074	0.030	0.030		
	D	0.101	0.030	0.032		
	P	36.5	0.0	6.7		
3–4	H	0.068	0.028	0.023		
	D	0.080	0.033	0.022		
	P	17.6	17.9	4.3		
4–5	H	0.086	0.057	0.065		
	D	0.105	0.082	0.102		
	P	22.1	43.9	56.9		
5–6	H	0.114	0.109	0.108		
	D	0.124	0.121	0.115		
	P	8.8	11.0	6.5		
0–2	H	0.061	0.045	0.044	0.042	0.026
	D	0.053	0.042	0.041	0.040	0.022
	P	13.1	6.7	6.8	4.8	15.4
2–4	H	0.035	0.013	0.011		
	D	0.042	0.013	0.011		
	P	20.0	0.0	0.0		
4–6	H	0.049	0.036	0.040		
	D	0.055	0.046	0.054		
	P	12.2	27.8	35.0		
0–6	H	0.0151	0.0078	0.0069		
	D	0.0159	0.0081	0.0070		
	P	5.3	3.8	1.4		

[1] H — Divisions per hour calculated from the frequent-observation data.
D — Divisions per hour calculated from the daily-observation data.
P — Difference between H and D expressed as a per cent of H.

It can be concluded that a fairly reliable description of the time course of the delay can be obtained with daily observations, if two successive division intervals are used and very low doses avoided, while the over-all effect can be measured with quite good accuracy even at low doses.

Variation between descendants from the same irradiated animal. An experiment was carried out in which the 4 products of the second division of each irradiated animal were isolated separately, and their behavior followed by daily observations. The dose (6800 ergs/mm^2) of ultraviolet from the germicidal lamp was sufficient to cause considerable death.

TABLE 2

Distribution of death to the 4 sublines from the first two divisions of ultraviolet-irradiated animals

CLASS [1]	FREQUENCY	EXPECTATION WITH RANDOM DISTRIBUTION
4	3	0.3
3	1	2.2
2	6	6.9
1	4	9.6
0	10	5.0

[1] This column shows the number of sublines which died after the second but before the 6th division.

Thirty irradiated animals were isolated, of which 24 survived at least two divisions. The 4 products of the second division of each of these surviving animals were carried as daily isolation lines. However, a number failed to survive to the 6th division, death usually occurring between the 2nd and the 4th division. The distribution of these late deaths is shown in table 2, together with the expected distribution on the hypothesis that death was randomly distributed to the sublines.

A chi-square test showed that the departure from expectation was significant and due to an excess of cases in which the 4 sublines were alike. Thus the sublines are correlated in viability.

Do the sublines tend to be alike with respect to the interval, after the second, which is longest? Unfortunately, the data do not give a clear answer, since in 55 of the 65 surviving sublines the 4th interval was the longest leaving too few in other intervals to be adequately tested. All that can be said is that different sublines can differ in which interval is the longest, and there is no clear evidence that they are correlated at all.

A variance analysis was performed upon the data to find out whether there was any connection between the sublines in the length of the period between the 2nd and 6th division. The data are shown in table 3 together with the comparison of the variance for differences between the sublines with that for differences between irradiated animals. The latter is significantly higher than the former, indicating that the sublines are correlated with one another.

It can be concluded that the 4 products of the second division are correlated with one another in respect to survival and time between the 2nd and 4th divisions. However, the connection is not absolute so that some sublines may die while others survive. The 4th interval may be the longest in some sublines and the 3rd or 4th in others, and there may be differences of several days between sublines in the time between the 2nd and 6th divisions.

Distribution of delay for various doses and wave lengths. Figures 1 and 3 show that the distribution of the delay to various division intervals is not quite constant with dose. The late period becomes longer relative to the rest as the dose increases. The relationship is brought out in figure 5 in which data from all ultraviolet experiments are plotted. For this purpose, the ratio of the average time between the 2nd and 4th divisions to the average time to the 6th is plotted against the average time to the 6th division. The data show considerable scatter but fall within a band of values with no evidence that one wave length is different from any other. The conclusion can be drawn that the distribution of delay

is the same for all wave lengths, or at least not greatly different.

Wave-length dependence of the total delay. The apparent lack of wave-length dependence in the distribution of delay

TABLE 3

Time between the second and sixth division for ultraviolet-irradiated animals in which the four products of the second division were kept separately. The times in the body of the table are in days. The calculations for the analysis of variance and the correlation coefficient were carried out with the reciprocals of these times.

IRRADIATED ANIMAL	SUBLINE			
	a	b	c	d
2	6.33	6.33	5.58	6.67
3	died	died	12.50	17.17
5	died	4.67	5.00	5.67
6	died	died	20.00	21.00
7	died	9.00	14.25	10.58
8	8.17	6.88	7.00	5.50
10	4.25	5.00	5.67	5.25
11	died	9.75	died	9.50
13	8.75	8.50	8.88	8.50
17	7.33	10.00	8.58	9.00
19	3.67	4.33	4.33	3.67
20	died	died	19.67	11.50
21	died	died	died	12.17
23	10.00	13.00	16.50	9.67
24	8.67	14.67	9.00	10.25
25	died	13.33	died	6.50
26	5.25	6.50	4.67	4.75
27	died	5.67	5.67	5.00
28	4.33	5.75	6.50	4.50
29	died	died	12.25	12.67
30	died	8.50	8.75	9.17

Analysis of variance

ITEM	MEAN SQUARES	DEGREES OF FREEDOM	VARIANCE RATIO	P
Between irradiated animals	0.05946	20	121	< 0.01
Within irradiated animals	0.00049	44		
Coefficient of correlation (ρ) 0.97.				

to the various division intervals is in contrast to the distinct wave-length dependence of the total effect. Curves of the form $y = ae^{-bx}$ were fitted by the least-squares method to the data. In this equation y is the reciprocal time to the 6th division, x is the dose of ultraviolet in ergs/mm^2, b is a constant which measures the effectiveness of the wave length and is assumed to be different for each wave length, and a is a constant assumed to be different for each experiment.

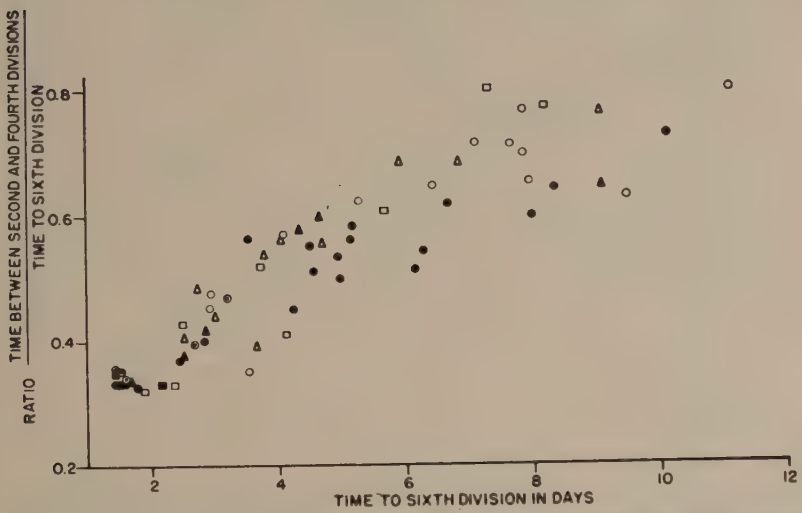

Fig. 5 The ratio between the time from the second to the fourth division and that from irradiation to the 6th division plotted against the time to the 6th division in days. All ultraviolet data are included.

● — germicidal
⊙ — 3022 Å
○ — 2804 Å
△ — 2650 Å
▲ — 2537 Å
□ — 2378 Å
■ — 2250 Å

We are indebted to Mr. Moshman of the Mathematics Panel of Oak Ridge National Laboratory, for carrying out the fitting procedure. The values of b are plotted in figure 6. The curve resembles to some extent the absorption spectrum for many proteins in having a maximum at about 2800 Å and a minimum around 2600 Å. The value of b for 2250 Å was 0.23, but this low value was probably spurious since the ani-

mals were rapidly immobilized and sank to the bottom of the tube. Thus they were protected by absorption in the overlying fluid which would be especially great at this wave length. Therefore, the point was omitted from the figure.

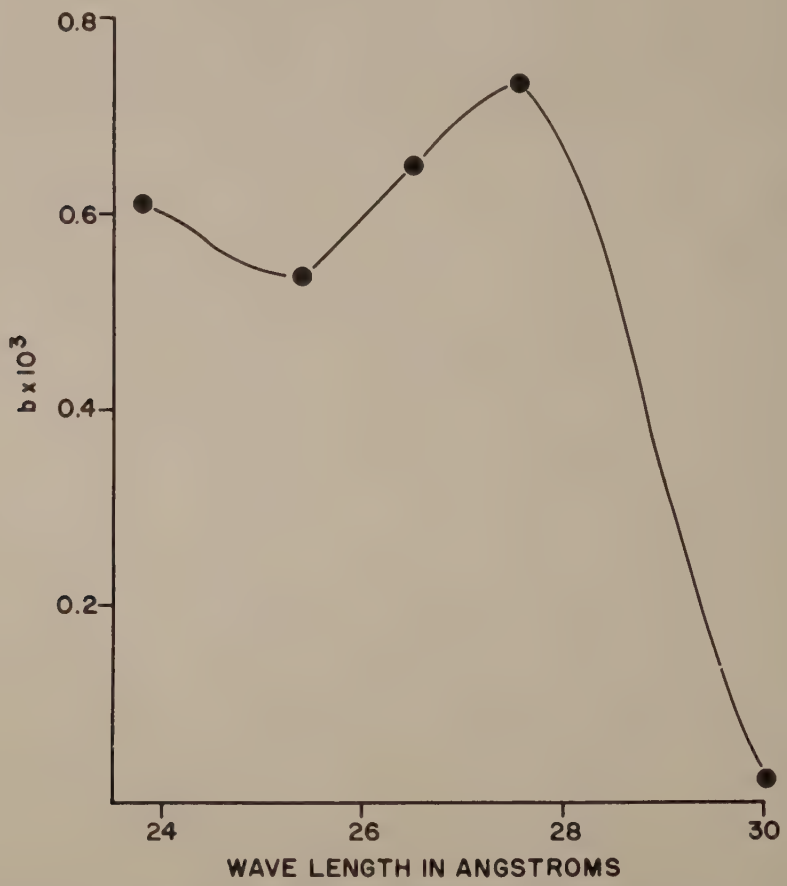

Fig. 6 The values of b determined by fitting curves of the form $y = ae^{-bx}$ to the data for various wave lengths of ultraviolet where y is the reciprocal time to the 6th division and x is the dose in ergs/mm^2.

Effect of temperature after irradiation. Two experiments were performed in which the effect of temperature in the first one or two days after irradiation was tested. The idea behind these experiments was that a slowing down of the

first one or two divisions might permit some recovery to occur, and so reduce the effect on later divisions. At least in part, this appears to have been the case.

Two experiments were performed, both with the germicidal lamp. In one, a group of paramecia were irradiated, divided into two groups, and isolated. One group of isolation lines was kept at 14°C. for 24 hours and then at 26°C. for the rest of the experiment. The other group was kept continuously at 26°C. Unirradiated controls were exposed to these two temperatures in the same way. In a second experiment, two additional groups were set up, one for 24 hours at

TABLE 4

Effect of low temperature after irradiation upon division delay by ultraviolet

EXPER.	TEMPERATURE (°C.)	DAYS AT TEMPERATURE	NO. OF UNIRRADIATED ANIMALS	AVERAGE TIME IN DAYS 0–2nd division		2nd–4th division
				Uncorrected	Corrected	
1	26	continuous	56	1.7	1.7	3.9
	14	1	64	2.3	1.6	1.4
2	26	continuous	67	1.7	1.7	2.4
	20	1	63	2.0	1.7	2.7
	14	1	42	2.1	1.5	1.7
	14	2	65	2.9	1.5	1.7

20°C. and one for 48 hours at 14°C. At the end of these periods, the temperatures for all groups were raised to 26°C. for the rest of the experiment. Transfers and observations for division were made daily in both experiments.

The data are summarized in table 4. Average times in days between irradiation and the second division and between the 2nd and the 4th divisions are compared as measures of the early and late effect. It is clear that the average time to the second division increases as the temperature decreases, while the time between the 2nd and 4th divisions decreases. The latter interval occurred in all lines of descent while the animals were at 26°C., so that these times are comparable for all groups. However, at least a part of the interval from irradiation to the second division oc-

curred at low temperature. Therefore, this interval would be lengthened by the low temperatures regardless of whether or not the animals had been irradiated. A correction for the retardation of division by temperature was made in the following way. It was assumed that one day at low temperature was the equivalent of a fraction of one day at 26°C. This fraction was the ratio between the average number of divisions per day in the controls at the temperature in question and the average number in the controls at 26°C. When this correction is applied, it appears that low (14°C.) temperature had no effect upon the ultraviolet-induced delay in the first two divisions, although it had a marked effect upon later intervals. Neither the somewhat higher temperature of 20°C. nor increasing the time at 14°C. from one to two days had any effect. If the action of temperature is due to a prolongation of the time during which recovery can occur, then it appears that the prolongation must be more than some threshold amount to produce an effect, but it must occur during the early part of the interval between irradiation and the second division.

X Rays

An hourly observation experiment was carried out using 7 doses of X rays — 34, 68, 140, 175, 210, 240, and 280 kr, and a control. Thirty animals were isolated for each dose group, and at least 27 survived from all save the 280-kr group, of which only 9 survived. The other 21 in this group died within 24 hours without dividing. Hourly observations were continued until all but two lines of descent in the highest dose group had completed at least 6 divisions. These two lines reached the 6th division following observation intervals of 12 and then 24 hours.

Results for 6 of the dose groups are shown in figure 7. A comparison with figure 1, in which the data are plotted in the same way, shows that X rays have relatively more effect than ultraviolet upon the first division, and relatively less upon later ones. However, the pattern of the effect is

the same except for this difference. The highest dose of X rays appears to have had about the same effect upon later intervals as the lowest dose of ultraviolet. The two sets of curves cover rather completely the available range of effect A tendency for the peak of the late effect to shift toward earlier division intervals was mentioned in the section on ultraviolet. This is further indicated by the data for low doses of X rays which suggest that the 4th and 5th,

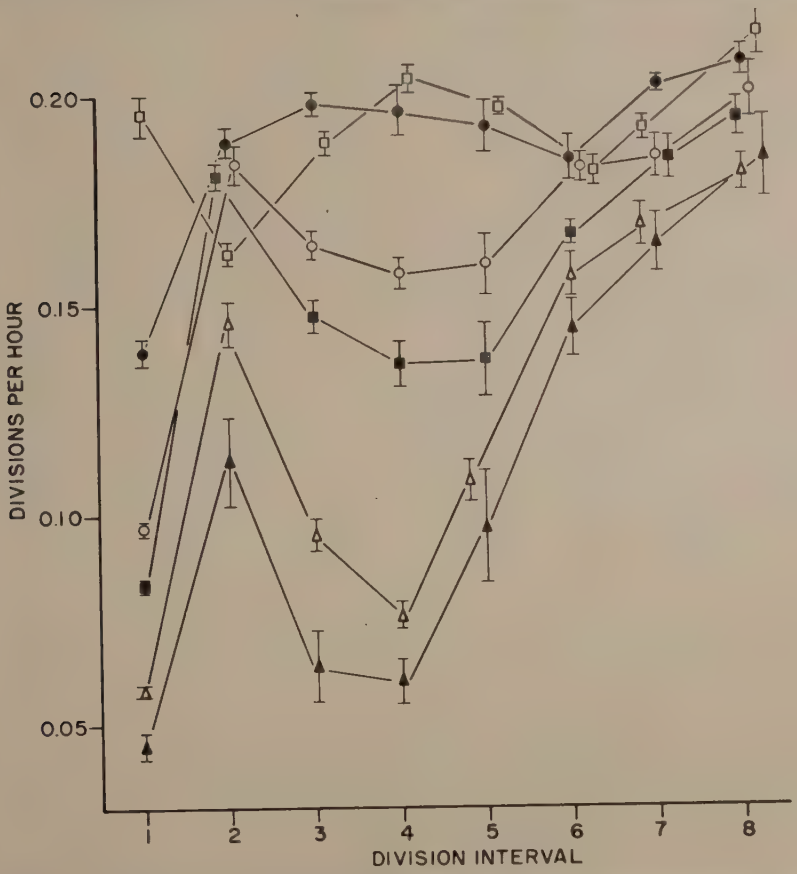

Fig. 7 Data from the X-ray experiment with hourly observations for mean reciprocal time for successive division intervals.

□ — none
● — 34 kr
○ — 140 kr
■ — 210 kr
△ — 240 kr
▲ — 280 kr

rather than the 3rd and 4th, are the longest intervals after the first. The data agree with the ultraviolet finding that recovery is almost complete by the 7th division at all doses.

No clear evidence for a negative correlation between the duration of the 3rd and 4th intervals was found nor were long cessations of division observed. Also, no death occurred after the first division. The failure to observe such phenomena is not surprising, however, since the highest dose of X rays corresponded in its effects on the later intervals to the lowest dose of ultraviolet. From the similarity of figures 1 and 7, it may be guessed that these phenomena are not observed because X-irradiated material does not survive at sufficiently high doses.

Thus it appears that X and ultraviolet radiation have the same pattern of effect upon division in *Paramecium* but that the early effects, i.e., delay in and death before the first division, are relatively more important with X rays than they are with ultraviolet.

The dose curves for the various intervals are shown in figure 8 in comparable manner to figure 2 for the ultraviolet. The curve for the first interval is very similar in form to that for ultraviolet; and, since about the same range of effect is covered, suggests a basic similarity between the delay produced by the two types of radiation. The curves for later intervals cover such a small range of effect that they are not comparable to those for ultraviolet. It is possible that the ultraviolet curves would be similar, but no data are available in this range.

The curve relating the rate for the period to the 6th division to dose is shown in figure 4. The difference between this and the corresponding curve for ultraviolet is obvious, probably reflecting the greater relative importance of retardation of the first division in the case of X rays.

Nitrogen mustard

Several experiments were carried out using the radiomimetic substance, nitrogen mustard. In two experiments, *P.*

aurelia stock 90 and *P. caudatum* were both used and observations were made hourly. In one experiment, stock 90 was used and observations were made daily.

Attempts to vary the dose by increasing the concentration of the mustard or increasing the time of exposure do not appear to have been very successful. A maximal effect was obtained after exposure to a relatively low concentration and

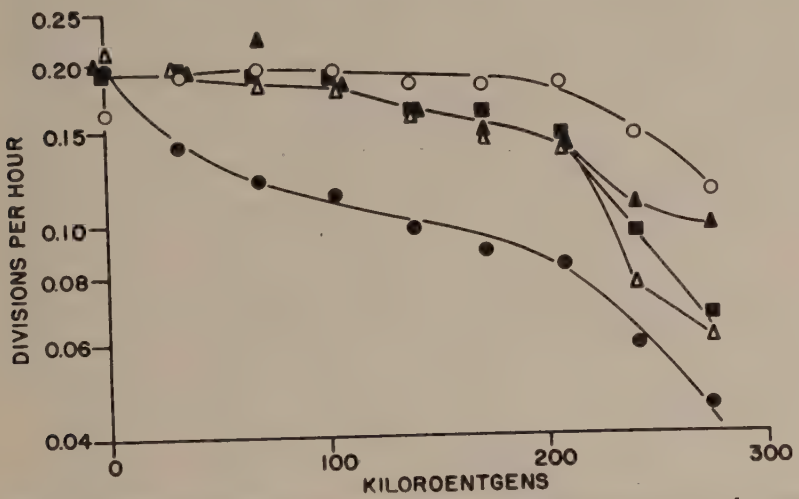

Fig. 8 Divisions per hour for given division intervals plotted on a log scale against dose of X rays in kr.

● — 1st interval ■ — 3rd interval ▲ — 5th interval
○ — 2nd interval △ — 4th interval

neither doubling the concentration nor increasing the exposure time from 10 to 15 minutes appears to have had much further effect (fig. 4). It may be that this represents a true maximum of the effect that can be produced with mustard, but it seems more probable that the amount of the substance which could enter the animals under our conditions was somehow limited.

The results of one of the two experiments with hourly observations are plotted in figure 9. Nitrogen mustard resembles X rays in having its most pronounced effect upon the first interval. However, it also affects later intervals.

This is clearly shown in figure 9 for *P. caudatum* but is not clear for *P. aurelia,* since not only the treated animals but the controls showed a considerable decrease in rate during

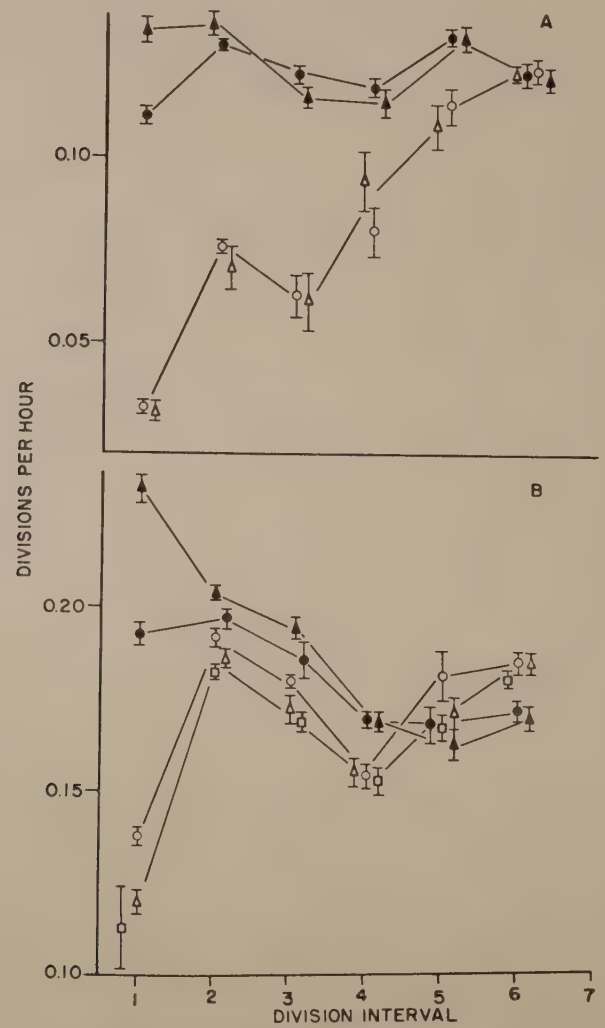

Fig. 9 Data for divisions per hour for successive division intervals for one of two nitrogen mustard experiments. (a) *P. caudatum,* (b) *P. aurelia.*

▲ — none
● — 0.05 mg/ml for 10 min.
○ — 0.50 mg/ml for 10 min.
△ — 1.35 mg/ml for 10 min.
□ — 1.35 mg/ml for 15 min.

these intervals. It must be remembered that the decrease in the controls did not completely coincide in time with that in the treated, so it may have been due to different causes. Nonetheless, these data do not demonstrate clearly any effect upon later intervals for *P. aurelia.* In the daily observation experiment, three lines of descent survived a treatment with 2 mg/ml of mustard for 10 minutes. All three clearly divided at low rate for several divisions before recovering so that the failure to find an effect upon later divisions in the hourly observation experiments probably is due to the small total effect which was produced. The *P. caudatum* data show some evidence that the second interval is shorter than either the first or third. However, the 4th interval is also short, suggesting that the distribution of delay may not be the same in this stock of *P. caudatum* as in stock 90 of *P. aurelia.*

In general, the data for nitrogen mustard suggest that the basic pattern of division delay by this substance is much the same as for X radiation. However, the substance has proved difficult to use because the paramecia are killed at doses which produce relatively little effect upon division.

DISCUSSION

The major feature which appears, at first glance, to distinguish division delay in *Paramecium* from that in other organisms is the marked effect upon later division intervals. However, it is not clear that this distinction is real. Many investigations with other organisms have not extended over a sufficient time to detect effects on the later intervals. Moreover, the X-ray experiments with *Paramecium* show that considerable effect may be produced upon the first interval without producing more than a slight effect upon later intervals.

Various pieces of evidence suggest that delay in later division intervals may occur in other organisms. Several cases of such effects have been reported for cleavage delay in the eggs of marine invertebrates. Kimball (in press) may

be consulted for review. However, these effects have always been very small in comparison to those upon the first cleavage after irradiation. Blum and Price ('50) have shown that these effects on later cleavages are due to the recovery process extending over several divisions.

It seems possible that a similar recovery from the effect upon the first division occurs in *Paramecium,* but is obscured by a more marked effect of another kind. It is possible that this latter effect concerns growth processes and so does not occur at all in the early cleavages of eggs, for in them little or no growth takes place. However, a number of workers (see Giese, '49, for review) have reported that sufficiently high doses of radiation lead to irregular cleavages. This might be expected if there were considerable delays in later cleavages, but these delays were of different duration for different blastomeres.

In irradiated vertebrate tissues, Luther ('48) recognizes three periods based upon counts of the number of cells in mitosis: (1) a fall in mitotic count to zero or nearly zero, (2) a rise in the count to a peak, usually less than the controls, (3) a secondary fall in the count from which a slow recovery takes place provided the dose is not too high. The primary fall in count probably is due to the inhibition of the first mitosis after irradiation. The secondary fall is interpreted by Luther ('48) as the result of the death of many of the cells which undertake the first mitosis. This interpretation is based upon the large number of abnormal mitoses which are seen at the time of the first peak in the mitotic count. Certainly, it appears highly probable that cell death accounts for much of the secondary drop. Nevertheless, it remains possible that a part of the drop is due to effects on mitoses later than the first in those cells which survive.

Thus, it is possible that a considerable delay in later intervals is not just a special response of *Paramecium,* and perhaps other ciliates, to radiation but is of more widespread occurrence. However, the evidence on the matter is not yet decisive. It may be pointed out that *Paramecium* is favorable

for the investigation of such delayed effects not only because individual lines of descent may be followed but also because, as Kimball ('49) has pointed out, little or no injurious effect of gene mutations and chromosome aberrations is to be expected as long as the paramecia reproduce vegetatively. Thus death from genetic causes is avoided, and phenomena requiring higher doses can be investigated.

There is considerable evidence that delay in the first division is a result of an inhibition at some one stage in the mitotic process. Thus Carlson ('40, '42, '49) has shown for the grasshopper neuroblast that the cells proceed at nearly normal rate up to a mid-prophase and then stop or even revert to an earlier stage. Rather similar findings are reported by Henshaw ('40b) for the sea urchin egg. A number of other investigators have also found that cells are stopped just before reaching mitosis or during prophase. Carlson ('42) may be consulted for further discussion. Such findings suggest that radiation acts to interfere with some particular key step in the mitotic mechanism.

No critical evidence is available on this matter for *Paramecium*, and it is possible that a similar situation holds for delay in the first division. However, it appears probable that a different mechanism is involved in the delay in later cleavages. In the case of prolonged cessation of division, the paramecia increase in size very little, if at all, following the division preceding the long interval. Rather, they become gradually smaller and increase in size only at the very end of the interval. One gets the impression that the general growth of the cell has been inhibited and that delay may come about from the failure of the cell to reach the necessary size. While this interpretation appears quite possible, it must be emphasized that the control of the onset of division is a complex matter and that increase to a certain size is not always a necessary feature. Extremely small paramecia can divide under certain circumstances, and so it is not clear whether inhibition of growth leads to division delay or the two are different symptoms of the same cause.

As Lea ('47) points out, it is easiest to think of division delay by radiation as the result of the destruction of some substance necessary for division or the production of some substance inhibitory to it. Recovery can, then, be thought of as resynthesis of the destroyed substance or destruction of the inhibitory substance. It is always possible that more than one substance is involved.

Lea ('47) builds up an hypothesis of one such substance whose resynthesis (or destruction) represents recovery and shows that such an hypothesis can account reasonably well for the quantitative and qualitative aspects of delay in the first division, especially as exemplified by Henshaw's data on the sea urchin egg. Apparently he assumes that once division has occurred the recovery is complete. It would be possible to accept this simple hypothesis to account for all or part of the delay in the first division in *Paramecium;* but it cannot account, without modification, for delays in later divisions than the first. Blum and Price's ('50) finding that recovery in the sea urchin egg extends over several divisions suggest that it may not apply in unmodified form even to delay in the sea urchin egg.

Even though this simple mechanism cannot account for all the division delay phenomena in *Paramecium,* it might be possible that a single primary effect of the radiation is involved. Superficially, the complex distribution to various division intervals and the complex dose curves suggest multiple effects. However, it must be remembered that delay in division may be many steps removed from the primary action of the radiation. The destruction of some substance directly concerned in the first division might have secondary consequences upon later divisions even after the originally destroyed substance had returned to nearly normal concentration. Complex dose curves could result from a complex response of the cell to the concentration of some substance produced or destroyed by the radiation rather than to multiple primary effects.

Nevertheless, there is one important reason for believing that there are at least two primary effects: (1) an effect upon the first interval after irradiation and (2) a later effect expressing itself as delay in the 3rd, 4th, and 5th intervals. This is the fact that X rays and mustard have a more marked effect upon the first interval, relative to the later ones, than does ultraviolet. If all three agents acted to produce the same primary effect, such a difference should not occur. The remarkable similarity in all other details of the X-ray and ultraviolet effects makes it difficult to believe that a different single primary effect could be involved in the two cases.

How is the effect of radiation upon the later intervals to be explained? The most noticeable feature is that the intervals which are affected are almost, though not quite, independent of dose. Only the degree of the effect, i.e., the rate at which the affected division intervals proceed, is dose dependent. This fact can be accounted for on the assumption that radiation destroys or reduces the ability of the cell to make some material which is required for division and is originally present in excess. As long as the cell makes this material at a low rate or not at all, successive divisions will serve to decrease the concentration per cell until finally the amount is reduced below that which is required for division. The original excess is apparently enough to allow the cell to divide two to three times, a number which would not be dose dependent. At lower doses, the substance might still be made but at a reduced rate so that two to three long intervals might occur, the length of each interval reflecting the time needed to just build up the material to the amount necessary for division. At higher doses, the cell might be incapable of making the substance at all. Thus the cell would cease to divide after depleting its original reserves and would not start to divide again until the recovery process has restored its ability to make the substance. The divisions after the interval of cessation would be expected to be fairly rapid since by then recovery should be well along. The length of the cessation period would be proportional to the time needed

for recovery and so would be dose dependent. It is obvious that "substance" as used here need not be a single chemical entity. It might, for example, be a whole group of enzymes, originally present in excess of the minimum needs of the cell. However, it could not be all the proteins of the cell; for after irradiation, enough new material is formed to give rise to 4 to 8 cells which are not noticeably smaller than normal. The radiation must prevent the cell from synthesizing some but not all its materials. This hypothesis also allows for, indeed makes probable, the existence of differences between species in the distribution of division delay. The amount of "reserve material" would determine the form of the distribution. The existence of such differences is suggested by the studies of Giese and Reed ('40) on various species and strains of *Paramecium*.

There is a considerable and convincing body of evidence that delay in the first cleavage in the sea urchin egg is due to an effect upon the nucleus (Blum et al., '50). As Kimball (in press) points out there is no critical evidence on this matter for *Paramecium*. Giese ('45) has reported action spectra for the effect of ultraviolet upon the division rate in *P. caudatum*. He uses two measures, time to the third division and time to recovery. Time to the third division for starved animals and to recovery for well-fed (and, presumably, for starved also) shows an action spectrum with a peak at 2650 Å. Time to the third division for well-fed animals shows a rather nondescript action spectrum with a small peak at 2804 Å. Giese ('39) also reports that 2650 Å ultraviolet has more effect upon later division intervals than does 2804 Å. Figure 6 in the present paper is the action spectrum for time to the 6th division (and so, presumably, to recovery) for well-fed animals. It resembles Giese's ('45) curve for time to the third division rather than that for time to recovery in well-fed animals. Also, figure 5 fails to give any evidence that the later division intervals are more markedly affected by 2650 Å ultraviolet than by 2804. It should be emphasized that only relatively large differences

between wave lengths in the effect upon the first division would be detected by this method. We are unable to account for the discrepancies between our findings and those of Giese unless, perhaps, there are differences in the species used.

In any case, it does not appear that a demonstration of an association of a nucleoprotein type action spectrum with the later parts of the delay phenomenon gives a strong presumption that the nucleus is involved. This type of spectrum could as well be due to absorption in the nucleoproteins of the cytoplasm. The fact that an effect lasts for several divisions does not point strongly to the nucleus when complete recovery occurs within 6 to 8 divisions. Further discussion of this matter is given by Kimball (in press).

SUMMARY

1. Division delay by ultraviolet, X rays, or nitrogen mustard in *Paramecium* is shown to involve a retardation not only in the first interval after treatment but also in later intervals.

2. The effect upon the first interval is more marked, relative to that upon later intervals, with X rays and nitrogen mustard than it is with ultraviolet.

3. At low doses, the 3rd, 4th, and sometimes the 5th, intervals of a given line of descent are longer than the second interval.

4. At high doses, some one interval, usually the 3rd or 4th, is often very long, sometimes lasting as long as two or three weeks. When no interval is very long, two or more intervals are of intermediate duration.

5. Different treated animals and even different lines of descent from the same treated animal may differ in which interval is the longest.

6. Recovery of the normal rate is usually nearly complete by the 6th division.

7. The distribution of the delay to the various division intervals changes only slightly with dose.

8. There does not appear to be any marked effect of the wave length of ultraviolet used upon the distribution of the delay to the various division intervals, but the total effect shows an action spectrum with a small peak at 2804 Å.

9. The question of whether the effect upon later intervals is a special feature for *Paramecium,* and perhaps other ciliates, or is of general occurrence is discussed. It is concluded that the methods of study available for other organisms are not very favorable for its demonstration, but there is some evidence which is suggestive that it may be of general occurrence.

10. It is concluded that there are at least two different effects involved in division delay in *Paramecium;* a delay in the first division which may correspond to the inhibition of a particular stage in mitosis reported for other organisms, and an effect which is most readily explained as an inhibition of the synthesis of some substance necessary for division but originally present in excess.

11. It cannot be decided whether the nuclei, the cytoplasm, or both are involved in division delay in *Paramecium.*

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EFFECTS OF ANESTHETICS ON OXYGEN CONSUMPTION AND ON SYNAPTIC TRANSMISSION IN SYMPATHETIC GANGLIA [1]

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Pre-eminent among contemporary hypotheses concerning the mode of action of anesthetics on nerve cells is one which supposes that the depressant effects of these substances are due to an interference with cellular metabolism. This interference is assumed to result from inactivation of certain enzymes, which are as yet only partly identified. It is appropriate that this hypothesis receive extensive consideration and test at a time when considerable effort is being devoted to elucidation of the role of enzymes in controlling the chemical reactions and functional properties of living cells.

Experimental evidence that anesthetics interfere with metabolism has been obtained mostly from measurements of oxygen consumption. The observations of Quastel and his colleagues, and subsequent confirmations and extensions by other laboratories, have abundantly demonstrated that a wide

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variety of anesthetic substances are able to depress the oxygen consumption of excised brain tissue (e.g. Quastel and Wheatley, '32; Jowett and Quastel, '37; Jowett, '38; Fuhrman and Field, '43). Clues as to the enzymes affected have also been obtained (Michaelis and Quastel, '41; Grieg, '46; Persky, Goldstein and Levine, '50).

Although it has thus been established that anesthetics are able to retard the oxygen uptake of excised brain tissue, it is much less certain that the concentrations required for this effect are attained in the body during anesthesia. One difficulty is that the concentration of anesthetic in the body, or more specifically in the blood, has been measured for relatively few substances. The concentration of other anesthetics in blood can only be estimated by making the unsatisfactory assumption that it is equal to the dose per kilogram of body weight. It is recognized, however, that the actual concentration may differ from this estimate, due to slowness of absorption, continual destruction or excretion, and non-uniform distribution in the body. Moreover, when anesthetics are applied to excised brain tissue in concentrations selected in accordance with the best available information, depression of oxygen consumption is by no means universally observed (table 1). Further doubt concerning the fundamental significance of metabolic effects, even when observed at concentrations required for anesthesia, has been raised by Butler ('50) in a recent review. He points out that doses far below those required for surgical anesthesia produce well-defined depression of function (e.g. hypnosis in man). There is no evidence that these much lower concentrations have any effect on metabolism of nervous tissue.

In interpreting experiments on excised brain tissue, not only do problems arise concerning the concentration of anesthetic, but there also exists the possibility that the nerve cells themselves are significantly altered by removal from the body. When a tissue is excised, sliced or homogenated, and placed in an artificial medium containing little or no CO_2, it is possible that the metabolic system becomes more

TABLE 1

Inhibition of rate of oxygen uptake by excised brain tissue

A. Substances for which blood concentration is unknown

	ANESTHETIC DOSE PER KG	INHIBITION BY ANESTHETIC DOSE PER LITER
	gm	
Barbiturates		
Barbital	0.34 (e)	0% (1)
Ipral	0.16 (e)	0% (1)
Amytal	0.08 (e)	5% (1)
Luminal (phenobarbital)	0.20 (h)	2% (1), 15% (2)
Evipan	0.16 (h)	17% (2)
Ortal	0.09 (e)	41% (1)
Other anesthetics		
Avertin (tribromethanol)	0.30 (h)	31% (2)
Chloral hydrate	0.22 (a,h)	10% (2)
Chloretone (chlorbutanol)	0.20 (a)	20–50% (2,4)
Urethane	0.90 (a)	< 5% (2)

B. Substances for which blood concentration is known.

	ANESTHETIC DOSE PER KG	CONC. IN BLOOD DURING ANESTHESIA	INHIBITION BY CONC. EQUAL TO THAT IN BLOOD DURING ANESTHESIA
Pentobarbital	43 mg (1)	50 mg/liter (c,d)	About 10% (3)
Ether		1.3 gm/liter (b,f)	0–10% (1)
Ethyl alcohol	4cm³ (a)	4.5 cm³/liter (b)	0% (2)
Chloroform		0.3 gm/liter (b,g)*	Not determined**

(a) Sollman and Hanzlick ('28).
(b) Goodman and Gilman ('41).
(c) Fisher et al. ('48).
(d) Forbes et al. ('49).
(e) Swanson ('34) and Swanson and Fry ('40), quoted by Fuhrman and Field ('43).
(f) Jones, Baldes, and Faulconer ('50).
(g) Morris, Frederickson and Orth ('51).
(h) Various authors, quoted by Jowett ('38).

(1) Fuhrman and Field ('43).
(2) Jowett ('38).
(3) Jowett and Quastel ('37).
(4) Quastel and Wheatley ('34).

* Concentration of chloroform in blood of man may be considerably less than that stated (cf. g).

** Quastel and Wheatley ('32) found glucose oxidation was 34% slower in brains excised from mice which had been narcotized with chloroform than in brains removed from unanesthetized mice. They do not indicate whether the chloroform concentration was suitable for prolonged maintenance of reversible surgical anesthesia.

than normally sensitive to substances in the cellular environment. For example, injury may increase cellular permeability to anesthetics, or permit substances to escape from the cells so that part of the metabolic system becomes extracellular and directly mixed with the anesthetic. An example of such an enzyme-leakage has recently been observed by Zierler et al. ('53) in experiments on muscle. The procedure of comparing effects on function in vivo with effects on metabolism in vitro is thus based on the implicit and questionable assumption that cellular properties are not significantly changed by excision or by attendant alterations in environment.

The particular difficulties in interpretation described above are avoided when the oxygen consumption of the naturally-circulated brain is measured in situ. Under these conditions it has been shown that the oxygen uptake of the brain is slowed by increasing the depth of pentothal anesthesia in monkeys, dogs, and humans (Schmidt, Kety and Pennes, '45; Homburger et al., '46; Himwich et al., '47). While avoiding uncertainties related to dosage and the physiological state of neurons, these observations present other difficulties in interpretation because of the spontaneous activity of brain cells. Evidence has been obtained by electrical recording that this spontaneous activity decreases with deepening anesthesia (e.g. Derbyshire et al., '36; Gerard, Marshall and Saul, '36; Beecher and McDonough, '39; Jarcho, '49). Moreover it is well known that changes in rate of oxygen consumption normally accompany changes in activity of nerve cells, whether the activity is initiated chemically, by electrical stimulation, or by excitation of receptors exposed to natural stimuli. The alterations of oxygen consumption may be considerable in some types of neurons even during moderate activity: for example, the rate of oxygen consumption of sympathetic postganglionic axons in the inferior cardiac nerve of the cat has been found to be twice the resting rate during stimulation at a frequency as low as 10 per second (Larrabee and Bronk, '52). In the brain, acceleration of

oxygen consumption has been observed when neuronal activity was increased by convulsant drugs or by direct electrical stimulation. (Observations in situ by Schmidt, Kety and Pennes, '45; Davies and Remond, '46; Davies, Grenell and Bronk, '48; and in vitro by McIlwain, '51). Accordingly, the observed slowing of brain oxygen consumption during anesthesia is not necessarily a consequence of direct interference with metabolic processes by the anesthetic; it may equally well be a secondary result of the reduction of spontaneous activity, the activity being depressed primarily through some non-metabolic mechanism. Concomitant reductions of spontaneous activity and of oxygen consumption have actually been observed during localized perfusion of the cerebral cortex with pentobarbital (Grenell and Davies, '50); this can be explained by either mechanism. Obviously, then, observations on the intact brain, as those on excised tissue, have so far failed to establish a metabolic basis for anesthesia.

Because of the inconclusive nature of investigations on the brain, we wish to present certain observations on excised sympathetic ganglia and on sympathetic nerve trunks. Sympathetic ganglia of mammals have recently proved useful structures for investigating fundamental problems concerning actions of anesthetics on neurons and on interneuronal synapses. Absence of internuncial neurons in these ganglia confers an anatomical simplicity, compared to the central nervous system, which greatly aids interpretation of experimental observations. Moreover, difficulties described above in interpreting metabolic measurements on brain tissue do not arise when sympathetic ganglia are studied after excision, as in our experiments. Under these conditions effects of anesthetics on rate of oxygen uptake and on synaptic transmission can be measured simultaneously, and the ganglionic neurons are at rest unless experimentally stimulated.

Numerous observations have indicated that the sensitivity of sympathetic ganglia to certain anesthetics is similar to that of the central nervous system. For example, synaptic

transmission through sympathetic ganglia of a cat is depressed when the animal is deeply anesthetized with ether or chloroform (Larrabee and Holaday, '52) or when ganglia are perfused with ether, chloroform, pentobarbital, or chloretone in concentrations approximating those in the blood during anesthesia. Similar concentrations in a bathing fluid depress transmission through excised rat and rabbit ganglia. These 4 anesthetics act selectively on synaptic mechanisms in these ganglia, since axonal conduction is not blocked at concentrations causing profound depression of synaptic transmission (Larrabee and Posternak, '52). Other agents, such as urethane and ethyl alcohol, have no such selective synaptic action and affect ganglionic neurons only in concentrations considerably exceeding those in the blood during anesthesia. Even these substances, however, produce readily reversible depression when raised to sufficient concentrations in perfused or excised ganglia.

METHODS

The experiments to be described in this paper were performed on superior cervical ganglia excised from rabbits during Dial anesthesia. One of these ganglia usually weighed between 5 and 10 mg and had a maximum transverse diameter between 1 and 1.4 mm. After excision, a connective tissue sheath was removed from the ganglion, which was then placed in a small plastic chamber filled with fluid (fig. 1). In this chamber the preganglionic nerve was drawn into a channel containing platinum stimulating electrodes, and the postganglionic nerve into another channel containing platinum recording electrodes; this permitted tests of transmission to be made at any time. Stimuli were always supramaximal. Changes in height of the postganglionic action potential thus served as a measure of changes in number of ganglion cells responding to preganglionic nerve impulses.

Except during measurements of oxygen consumption, bathing solution flowed continuously through the chamber, typically at a rate of about 4 cm^3/min. The solution was equi-

librated with 5% CO_2 and 95% O_2, and contained NaCl, 133 mM; KCl, 5.6 mM; $CaCl_2$, 2.22 mM; $NaHCO_3$, 16.2 mM; and glucose 5.5 mM (1 gm per liter). In a few of the later experiments a fluid was used which was similar to that just described, except for the addition of $MgCl_2$, 0.12 mM and NaH_2PO_4, 1.20 mM. These were added to provide a more natural bathing fluid, although we have no evidence that any change in the tissue resulted. The pH of both solutions

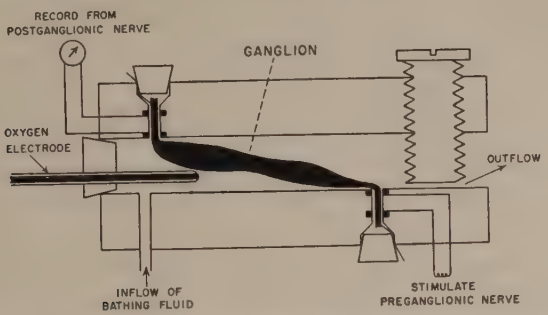

Fig. 1 Diagram of respiration chamber used in ganglion experiments. After inserting the ganglion (weight 5 to 10 mg), the chamber was closed with screw shown at right, leaving only enough opening for escape of bathing fluid. To control temperature the chamber was partly immersed in fluid in a small glass dish, which was in turn partly immersed in a large temperature bath. The circuit for the oxygen electrode was completed through a salt bridge to a calomel half-cell which is not shown in the diagram. The chamber was constructed of Lucite, the platinum stimulating and recording electrodes being embedded before drilling the channels for nerves and ganglion. The ganglion channel was about 2 mm in diameter.

was about 7.4. The solutions were equilibrated with the gases before adding calcium, magnesium, and glucose. $CaCl_2$ and $MgCl_2$ were prepared as 2 M solutions, checked with a hydrometer, and diluted to make isotonic stock solutions before adding to the bathing fluid. The reservoirs of bathing fluid and the chamber containing the ganglion were immersed in a temperature bath which was kept at 34°–37°C. except when lower temperatures are stated.

Principles of method for measuring rate of oxygen consumption. Rate of oxygen consumption was measured by stopping

the flow of bathing fluid for two minutes out of every 5 and measuring the rate of fall of oxygen concentration in the solution near the ganglion. The measurement was made with the oxygen electrode described in the next section. This type of respirometer, in which solution flows intermittently, has been called a "static respirometer" because the solution is at rest during the period of measurement. This contrasts with the "continuous flow respirometer," through which solution flows steadily, residual oxygen content of the fluid being measured at the exit (Carlson, Brink and Bronk, '50). A static respirometer employing the oxygen electrode was first used to measure oxygen consumption of a nerve trunk by Davies and Brink ('41).

When the flow of solution through the closed, static respirometer was stopped, the total oxygen content of the whole chamber obviously fell at a rate which was proportional to the rate of oxygen utilization by the tissue. Correspondingly, the oxygen concentration at any point within the respirometer, such as the point occupied by the tip of the oxygen electrode, also fell, at a rate which was faster the more rapidly oxygen was utilized.

For precise analysis of the properties of the static respirometer, it was assumed (a) that rate of oxygen consumption was independent of oxygen concentration down to a very low level, in agreement with observations of several investigators on various tissues (e.g., frog nerve, Gerard, '27; cat brain in situ, Davies and Remond, '46; rat brain homogenate, Elliott and Henry, '46; frog muscle, D. K. Hill, '48; yeast cells, Winzler, '41). It was also assumed (b) that oxygen concentration was sufficient to permit the maximum rate of uptake throughout the entire volume of tissue under observation. This assumption was probably correct for the smallest ganglia which we have studied, as shown by calculations utilizing formulae developed by various investigators for diffusion of oxygen into respiring tissue (cf. Höber, '45) and data obtained in this laboratory on the approximate rate of oxygen consumption by sympathetic ganglia. However, the largest ganglia may have had a core of tissue not adequately supplied with oxygen. Analysis indicates that this could not prevent changes in rate of respiration from being caused by anesthetics although it might reduce the apparent magnitude of these changes in

the largest ganglia. This is not, however, a matter for serious concern in view of the prolonged survival of even the larger ganglia, the uniformity of our results on large and small ganglia, and the contrast to be described between the lack of effects on oxygen consumption by certain concentrations of anesthetics and the large changes produced by other means.

Adopting assumptions (a) and (b) of the preceding paragraph, FitzHugh ('51) has shown that the average rate of utilization of oxygen by the tissue (q) is directly proportional to the rate of fall of oxygen concentration (dc/dt) at any point within a static respirometer under either of two sets of conditions. (i) One set of conditions is satisfied if measurement of dc/dt is made sufficiently long after stopping the flow of bathing fluid so that certain initial transients are over and dc/dt has become constant. In other words, the limiting value of dc/dt as t approaches infinity is rigorously proportional to q. Experimentally, it was sometimes impossible to reach the limiting value precisely, but we have approached it closely by measuring dc/dt during the second minute after stopping the flow. This was satisfactory, since dc/dt remained relatively constant after the first half minute. (ii) The second set of conditions revealed by the analysis specifies that geometrical relationships be kept fixed within the respirometer and that the measurement of dc/dt always be made at the same time after stopping the flow of bathing fluid. Under these circumstances dc/dt is rigorously proportional to q, even if the limiting value of dc/dt referred to in condition (i) is not reached, provided however, that changes in experimental variables alter the rate of oxygen uptake in all parts of the tissue by the same proportion. In our experiments the tissue of a ganglion may not have behaved with such strict uniformity, but compliance with conditions (ii) was nevertheless desirable to minimize errors resulting from failure to conform rigorously to condition (i). Therefore the period of measurement was timed by a synchronous motor which also turned the flow of bathing fluid off and on.

Accordingly we have assumed that the rate of oxygen utilization by the tissue (q) was directly proportional to the rate of fall of oxygen concentration observed during the second minute after stopping flow. Thus:

$$q = p(dc/dt) \qquad (A)$$

The proportionality factor (p) is constant for a particular experimental preparation, but is in general an unknown function of the position and time at which dc/dt is measured and

of the position and dimensions of the tissue. Therefore we have not attempted to measure absolute rates of oxygen uptake by this method, but only to compare the relative rates for a particular ganglion held in a fixed position in the respirometer under various experimental conditions. Thus:

$$\frac{q_1}{q_2} = \frac{(dc/dt)_1}{(dc/dt)_2} \qquad (B)$$

where the subscripts 1 and 2 refer to the two conditions under which oxygen uptake is to be compared.

Oxygen concentration was measured with a platinum electrode whose tip lay close to the ganglion (fig. 1). This electrode was polarized with respect to a calomel electrode which was also in contact with the bathing fluid. With a suitable polarizing voltage the electrode current was directly proportional to oxygen concentration (Davies and Brink, '42):

$$i = k\ c \qquad (C)$$

where k is the calibration factor which measured the sensitivity of the oxygen electrode. Substituting in equation (B):

$$\frac{q_1}{q_2} = \frac{(di/dt)_1}{(di/dt)_2} \Big/ \frac{k_1}{k_2} \qquad (D)$$

Thus the relative rates of oxygen uptake under two conditions (q_1/q_2) can be determined by measuring di/dt in each condition and the ratio of the calibration factors (k_1/k_2). Actually the calibration factor remained essentially constant under the conditions of our experiments so that only di/dt had to be measured, as discussed below.

Measurement of di/dt. The platinum wire in the oxygen electrode was insulated with glass except for the tip. The tip was thinly coated with collodion by dipping in a 5 or 10% solution of collodion in amyl acetate and drying in air. We found that such a coating prevented a progressive loss in electrode sensitivity which otherwise occurred when calcium and bicarbonate were both present in the solution. In addition, the coating rendered the electrode relatively insensitive to motion of the bathing fluid. This simplified the calibration of the electrode, as described in the next section. A collodion coating of

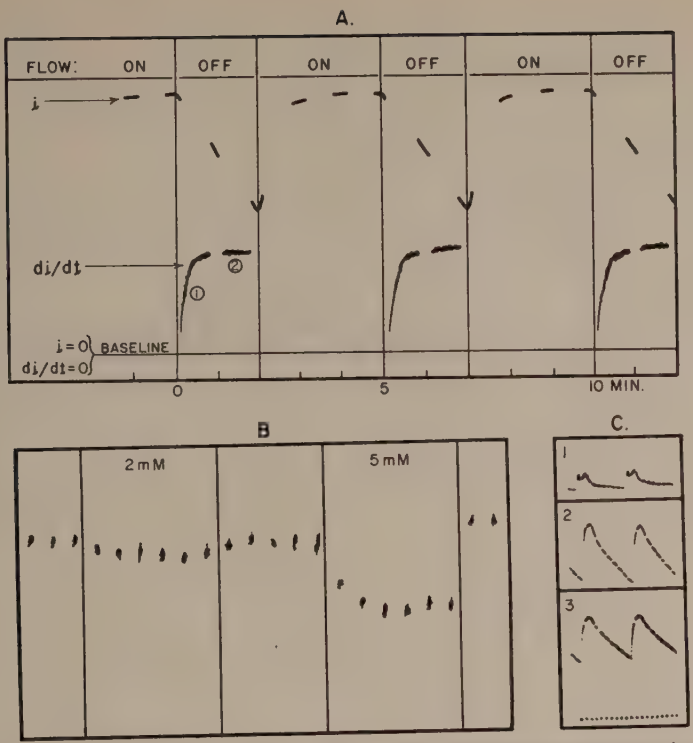

Fig. 2 A Diagram of a typical record, with time scale expanded for clarity. The recording microammeter was alternately connected to the output of the direct coupled amplifier in order to record oxygen electrode current (i) and to the output of the differentiating circuit in order to record rate of fall of electrode current (di/dt). The latter tracing was badly distorted during the first minute after stopping flow (at 1 in the figure) due to lag in the differentiating circuit and to delay in reaching a steady state of oxygen diffusion within the respirometer. During the second minute (at 2) the deflection was directly proportional to rate of oxygen consumption by the tissue. Irrelevant portions of the tracing of di/dt are omitted during periods in which solution was flowing. The tracing of i was useful for calibration, as described in the text. Amplification was adjusted independently for the two functions, to give adequate deflections without overlap of the tracings.

B Changes in rate of oxygen consumption when a ganglion was exposed to solutions containing chloretone. A photograph of an original record, but showing only the derivative of the oxygen electrode current during second minute after stopping the flow (corresponding to 2 in part A of this figure). Deflections above the baseline are thus proportional to rate of oxygen consumption. Measurements were made at 5 minute intervals.

C Oxygen consumption by a cervical sympathetic nerve trunk. In this case only the current, not its derivative, was recorded. Flow was stopped for 8 minutes out of every 10. Tracings were interrupted at one minute intervals. Record 1 shows relatively small disturbances produced by stopping and starting flow when no nerve was in the chamber. Record 2 shows depletion of oxygen by resting nerve while flow was stopped, and rapid restoration when flow was resumed. Record 3 shows slower depletion of oxygen in presence of 5 mM pentobarbital. The zero level for oxygen coincided with time tracing, shown only in Record 3. Time is indicated in minutes.

such a thickness that sensitivity to oxygen was reduced about 4 or 5 fold proved suitable for these purposes.

The oxygen electrode current was measured by allowing it to flow through a fixed resistor and amplifying the resultant voltage drop with a direct coupled amplifier. The resistor size was so chosen that the voltage drop never exceeded 150 millivolts.

Amplifier output, which was directly proportional to electrode current, was registered on a General Electric recording microammeter. In some experiments a simple differentiating circuit was connected to the output of the amplifier. This produced a signal which was directly proportional to the rate of fall of electrode current. When the differentiator was used the recording meter was switched back and forth between the output of the amplifier and the output of the differentiator (cf. fig. 2 A). Thus, during each minute, electrode current (proportional to oxygen concentration) was recorded for about 15 seconds and the derivative of this current for about 45 seconds. The synchronous motor which accomplished this switching also controlled the flow of bathing fluid through the chamber.

In experiments where the differentiating circuit was employed the average value of di/dt during the second minute after stopping flow was estimated by inspection of the record. When the differentiator was not employed, the drop in i was measured between two fixed times during the second minute. Thus the average value of di/dt between those two times became $\triangle i/\triangle t$. Inserting this in equation (D):

$$\frac{q_1}{q_2} = \frac{(\triangle i/\triangle t)_1}{(\triangle i/\triangle t)_2} \Big/ \frac{k_1}{k_2}$$

or since $\triangle t$ was constant:

$$\frac{q_1}{q_2} = \frac{\triangle i_1}{\triangle i_2} \Big/ \frac{k_1}{k_2} \qquad \text{(E)}$$

Calibration of the oxygen electrode. Equations D and E show that the absolute value of the calibration factor for the oxygen electrode did not need to be determined. It was sufficient to show that no significant changes in electrode sensitivity resulted from presence of any substances in the concentrations employed in our experiments. Since this was true, $k_1/k_2 = 1$ in equations D and E.

Test of constancy of calibration depended upon two circumstances. (1) The oxygen electrode was coated with collodion, so that the current through it was only slightly altered by flow of the bathing solution. This was shown by starting and stopping the flow in "blank" runs with no ganglion in the chamber. Therefore the calibration could be checked while the solution was flowing. (2) The flow was normally fast enough so that the tissue removed only a negligible amount of

oxygen from the moving solution. Therefore the concentration near the electrode was essentially the same as that in the reservoir of bathing fluid. Under these conditions, from equation (C):

$$k_1/k_2 = (i_{f1}/i_{f2})\ (C_{r1}/C_{r2}) \qquad (F)$$

where C_{r1} and C_{r2} are the concentrations of oxygen in the reservoirs under the two conditions being compared, and i_{f1} and i_{f2} are electrode currents observed with fluid flowing. Some of the experimental substances we employed were non-volatile and of negligible vapor-pressure. With these substances C_{r1} could be made equal to C_{r2} by careful equilibration of the solutions with oxygen. Then $k_1/k_2 = i_{f1}/i_{f2}$. Other substances had vapor pressures so high that appreciable amounts of oxygen must have been displaced from the solution. With these substances, allowances for reduction in C_r have been made in calculating k_1/k_2. These allowances were made by using available data on partial pressures of the experimental substances.

It was found that changes in electrode sensitivity were negligible. For this reason none of the determinations of rate of oxygen consumption were corrected for the small changes in electrode sensitivity which may have occasionally occurred. Accordingly we have plotted in our figures the observed values of di/dt (or of $\triangle i$) as per cent of control values in the absence of anesthetics. In a few cases relative values of k are similarly shown.

Observations on nerve trunks. For purposes of comparison, observations were also made on sympathetic nerve trunks excised from rabbits under Dial anesthesia. These nerves were placed in a plastic respirometer similar to that used for the ganglion experiments, but constructed to fit the nerve trunks employed. One end of the nerve was placed on stimulating electrodes in a fluid-filled channel and the other end on recording electrodes in a gas-filled cavity. Oxygen consumption of a nerve trunk was measured by stopping the flow of bathing fluid, usually for 6 to 8 minutes out of every 10 (fig. 2 c). Rate of disappearance of oxygen was determined during the last several minutes before restoring flow. The bathing fluid was the same as that used in ganglion experiments. $MgCl_2$ and NaH_2PO_4 were always included. All observations were at 33–34°C.

Advantages of oxygen-electrode respirometers. Respirometers employing the oxygen electrode have certain advantages over classical manometric respirometers, such as that of Warburg. (a) Possibly most important is the ease of working at natural pressures of CO_2. This substance is essential for normal maintenance of certain nerve properties (e.g., Lorente de No, 1947) and is utilized as well as produced in cellular metabolism, at least in some tissues (Evans, '44; Ochoa, '51). (b)The absence of a gas phase permits addition of

volatile agents, such as ether, chloroform or cyanide, without elaborate precautions against their loss, such as those described by Robbie ('48) for the use of cyanide in the Warburg apparatus. (c) The flow of bathing fluid supplies fresh solution almost continuously to the tissue. Under these conditions, the capacity for synaptic transmission and the rate of oxygen consumption remain reasonably constant in excised ganglia for at least 6 or 7 hours. (d) Composition of the fluid is easily changed. Therefore, when studying effects which are reversible, many different solutions may be applied to the same preparation during the course of an experiment (e.g., fig. 2 B). (e) There is no need for temperature control of the precision required in manometric methods.

A different type of oxygen-electrode respirometer, in which solution flows continuously, has recently been described by Carlson, Brink, and Bronk ('50). Their instrument has several advantages over ours: the absolute value of rate of oxygen uptake can be determined, oxygen consumption is measured continuously instead of intermittently, and greater sensitivity can be achieved. For our purposes, however, the static (or "intermittent flow") type of respirometer was considered more convenient, since it permitted recalibration of the electrode alternately with each respiration measurement, as described above. This was a valuable control in exploring the effects of a large number of substances, some of which might conceivably have altered the sensitivity of the oxygen electrode. In addition the static respirometer is simpler to construct than the continuous flow instrument, which requires a high-precision mechanism for driving a syringe.

OXYGEN CONSUMPTION OF RESTING GANGLIA

The first experiments to be described were designed to compare the influence of various anesthetics on the capacity of ganglion cells to respond to volleys of preganglionic impulses with effects on the *resting* rate of oxygen consumption. In order that the resting metabolism should be disturbed by only a negligible amount, the testing volleys of impulses were initiated infrequently, usually only two or three volleys every five minutes. In some experiments observations were also made during brief periods of rapidly repeated activity; adequate periods of recovery were then allowed before recording under resting conditions.

Anesthetics and alcohols. Time courses of typical experiments with chloretone and with chloroform are illustrated in figures 3 and 4. Reversible depressions of both transmission and rate of oxygen consumption were easily obtained with chloretone. When chloroform was used, transmission recovered completely after partial block caused by the more dilute

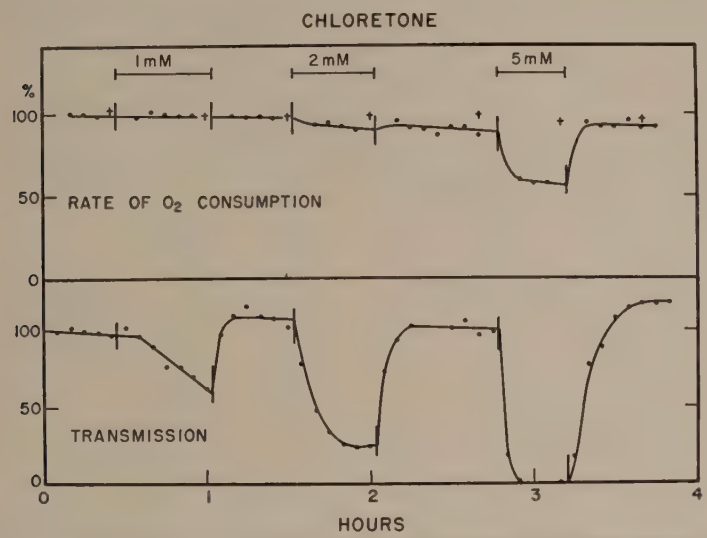

Fig. 3 Slowing of oxygen consumption and depression of transmission through an excised sympathetic ganglion bathed in various concentrations of chloretone. Transmission was measured by height of postganglionic nerve action potential, which served as an index of the number of ganglion cells responding to an occasional volley of presynaptic impulses. Presynaptic impulses were initiated by supramaximal stimulation of the preganglionic nerve. Crosses in the upper plot represent sensitivity of the oxygen electrode relative to sensitivity in control solution at beginning of experiment.

solutions; higher concentrations, however, had effects on both the postsynaptic response and on oxygen uptake which were partly or completely irreversible, at least during the recovery periods investigated. Despite the fact that oxygen uptake could be slowed by either chloroform or chloretone, our observations indicate that this oxidative interference was not responsible for the depression of response to preganglionic impulses. This is concluded from the fact that func-

tional depression was observed at concentrations lower than those which slowed oxygen uptake. This may be seen in the portions of figures 3 and 4 concerned with 1 mM chloretone and with .002% chloroform.

Similar results were obtained with all but one of the anesthetics which we examined: oxygen consumption could be depressed by sufficiently high concentrations, but was unaffected by the lower concentrations, which nevertheless pre-

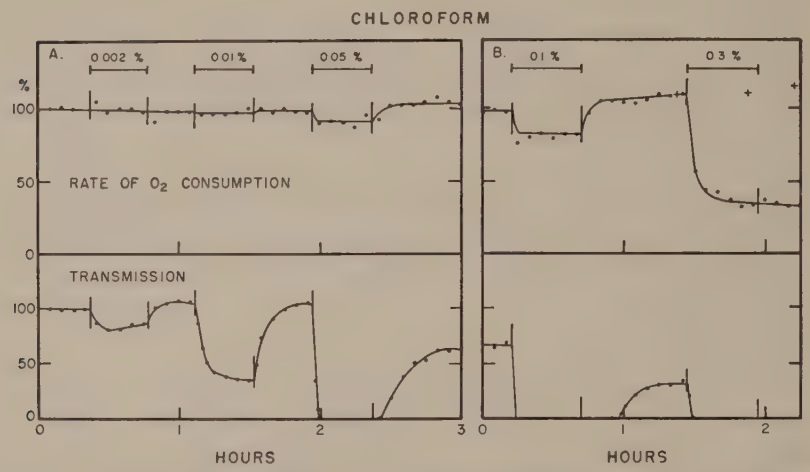

Fig. 4 Effects of chloroform on oxygen consumption and on transmission through a sympathetic ganglion. Data from two different preparations. In experiment B the graph of transmission starts at less than 100% because recovery had been incomplete from a previous exposure to 0.05% chloroform. Crosses in latter portion of part B indicate relative sensitivities of oxygen electrode in control and in chloroform solution. Other details as for figure 3.

vented many cells from responding to presynaptic impulses. Results of a number of experiments with chloretone, pentobarbital, and urethane are plotted against concentration in figures 5 A, B and C. Each point represents the height of the postganglionic action potential or the rate of oxygen consumption after at least 20 minutes' exposure to the experimental solution, expressed as per cent of control value in the absence of anesthetic. With pentobarbital, as with chloretone, synaptic transmission was markedly depressed or blocked by

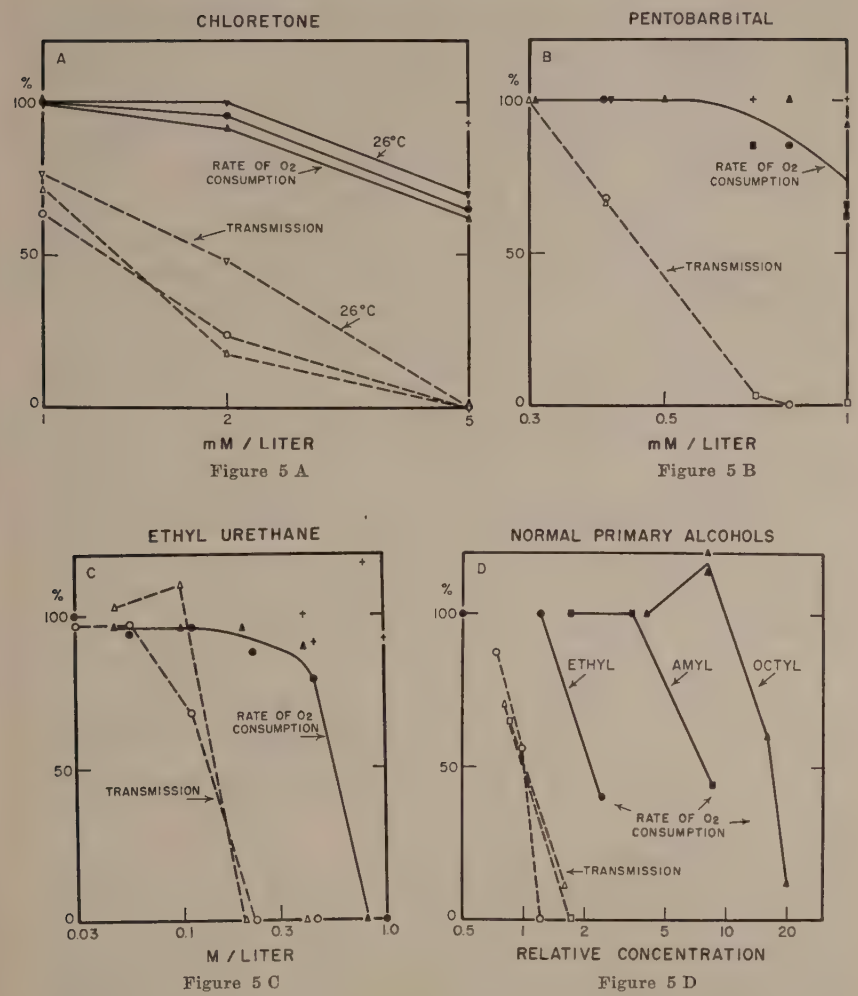

Figure 5 A

Figure 5 B

Figure 5 C

Figure 5 D

Fig. 5 Rate of oxygen consumption (filled symbols and continuous lines) and transmission (unfilled symbols and dashed lines) in ganglia exposed to various concentrations of the indicated substances. Experiments were conducted as illustrated in figures 3 and 4. Scales of concentrations are logarithmic. Crosses represent oxygen electrode sensitivity, expressed as per cent of sensitivity in control solution. One experiment with chloretone was at 26°C., all others at 34°–37°C. Observations on alcohols are plotted against a relative concentration scale in which unity equals the concentration of each agent required to depress the postsynaptic action potential by 50%. These unity concentrations were as follows: ethyl alcohol, 4.1 vols. % (700 mM); amyl alcohol, 0.059 vols. % (5.4 mM); octyl alcohol, 0.0025 vols. % (0.16 mM).

concentrations lower than those exerting measurable effect on oxygen uptake. Ethyl urethane, on the other hand, appeared to cause a small, scarcely detectable slowing of oxygen uptake, even in relatively low concentrations. This effect, which was not found with any other anesthetic, seemed unrelated to impairment of transmission, since it remained relatively constant over a range of concentrations in which transmission was progressively depressed; only at concentrations higher than those completely abolishing the postsynaptic response was there a considerable slowing of oxygen consumption. Observations on all of these substances thus showed that capacity for function could be impaired by an anesthetic independently of measurable effect on resting rate of ganglionic oxygen consumption.

A useful way of comparing the effects of a number of agents on function and on oxygen consumption is to plot the experimental results against a relative concentration scale, unity representing that concentration of each agent which depressed the postganglionic spike potential to one-half its control height. This has been done for three homologous alcohols in figure 5 D. It is evident that the difference between concentrations required for functional and for oxidative effects increased with molecular size among these alcohols.

All of our experimental data have been assembled in figure 6 on a relative concentration scale similar to that just described. Part A of this figure shows that with various substances progressive suppresion of the postganglionic action potential occurred chiefly in a 4-fold concentration range, extending from about one-half to about two on this relative concentration scale. This, therefore, is the functionally significant concentration range, to which attention should be directed in examining the metabolic data shown in part B of the figure. From part B it is obvious that only urethane, of all the anesthetics investigated, caused detectable slowing of oxygen uptake in the lower half of the functionally sig-

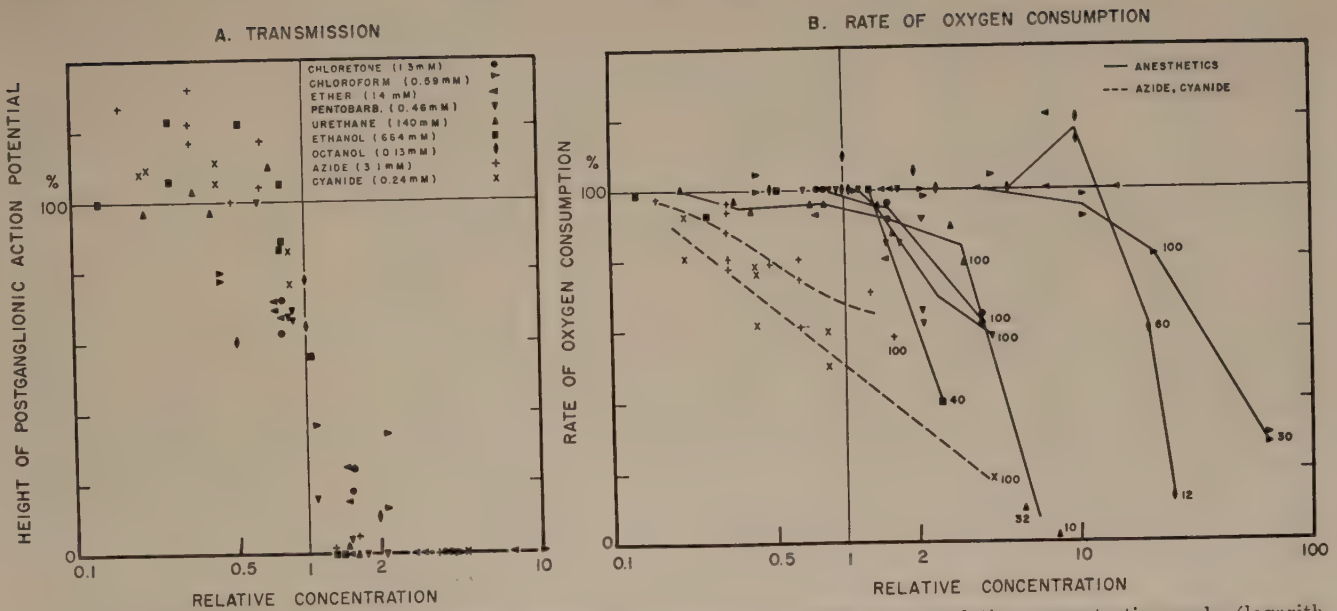

Fig. 6 Summary of observations on excised superior cervical ganglia, plotted on a relative concentration scale (logarithmic) in which unity equals the concentration of each substance required for 50% depression of height of postsynaptic action potential. List of unity concentrations is given in part A of figure. Note that filled symbols represent anesthetics, including alcohols; crosses and x's, azide and cyanide. In B continuous lines represent anesthetics and alcohols; dashed lines, azide and cyanide.

Degree of recovery of oxygen consumption after returning to control solution is indicated by numbers near some of the points in part B. For this purpose, rate of oxygen consumption was measured 15 or more minutes after removing the experimental substance, at a time when the rate had become approximately constant at a new level. Numbers indicate this rate as per cent of rate before application of anesthetic.

nificant range, and certain agents gave no evidence of oxidative effect until much higher concentrations were reached.

It was thus repeatedly demonstrated that depression of function by anesthetics could be independent of any measurable effect on resting rate of oxygen consumption.

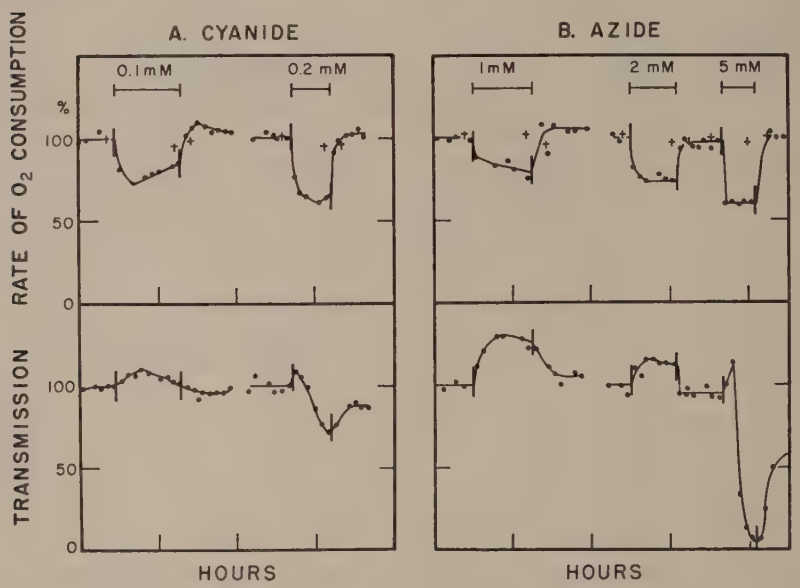

Fig. 7 Effects of cyanide and azide. Details as in figure 3. The lower concentrations of each agent retarded oxygen uptake considerably without blocking synaptic transmission. The increase in height of postganglionic action potential at these concentrations may have been caused either by an increase in number of ganglion cells discharging impulses or by an increase in size of action potential of each impulse; thus the increase is not necessarily due to a facilitation of synaptic transmission.

Other substances. In contrast to anesthetics, which depress transmission without retarding oxygen uptake, there are other substances and procedures which can conversely slow oxygen consumption to a considerable degree without immediately impeding transmission. One example is lack of oxygen itself, since transmission is unimpaired for many minutes after sudden ischemia makes oxygen unavailable to the tissues (Bronk, Larrabee and Gaylor, '48). The delay in de-

pression caused by lack of oxygen thus contrasts strikingly with the immediate impairment of function which usually followed application of an anesthetic.

In the present experiments it was found that less drastic, although considerable, slowing of oxygen uptake could be maintained for many minutes by suitable concentrations of cyanide or azide, without reducing the number of ganglion cells which responded to a volley of preganglionic impulses (fig. 7). Sufficiently high concentrations of either agent were, of course, able to depress the response. The contrast between cyanide and azide on the one hand, and anesthetics on the other, is further emphasized in figure 6. It is apparent that azide and cyanide considerably retarded oxygen consumption in the lowest concentrations which depressed transmission.

These observations thus indicate that anesthetics impair the functional properties of neurons by mechanisms other than those pertaining to the action of azide and cyanide. There is considerable evidence that the latter substances interfere with the cytochrome-cytochrome oxidase system (e.g. Winzler, '43). Our results are thus in agreement with evidence obtained by Michaelis and Quastel ('41), Grieg ('46), and Persky, Goldstein and Levine ('50) that anesthetics do not attack this part of the metabolic system. These authors propose that anesthetics affect dehydrogenases or flavoproteins. However, it is not obvious just how anesthetics could profoundly modify function by action on these enzymes, without the resultant metabolic disturbances being revealed in our experiments by changes in rate of oxygen consumption.

OXYGEN CONSUMPTION OF RESTING NERVE TRUNKS

In interpreting observations described above which demonstrate that synaptic transmission can be blocked by anesthetics without measurable effect on the oxygen consumption of resting ganglia, it must be remembered that the transmission process involves action in an entire sequence of structures, including presynaptic axons, the terminations of

these axons, the bodies and dendrites of the postsynaptic cells, and the axons of these cells. Moreover it has been shown that certain anesthetics block synaptic transmission in concentrations too low to interfere with axonal conduction (Larrabee and Posternak, '52). Although this selective action may be variously interpreted, one important possibility is that the effects of some agents in suitable concentrations are limited to structures in the synaptic regions, such as

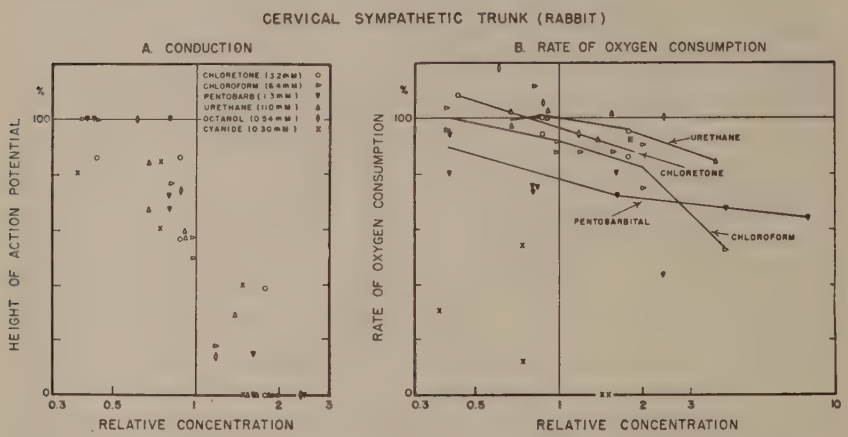

Fig. 8 Summary of observations on excised cervical sympathetic trunks, plotted on a relative concentration scale similar to that used in figure 6, except that unity in this figure represents the concentration of each agent required to halve the height of the B-fiber action potential of the nerve trunk. The action potential was elicited by a supramaximal stimulus and recorded after conduction for about 22 mm. Table of unity concentrations appears in part A.

axon terminations or cell bodies. It is conceivable that any particular structure might contribute so little to the total oxygen uptake of a ganglion that considerable changes in its metabolism would not be detected. The possibility thus remains that anesthetics block transmission by impairing oxidations in some small portion of the total neuronal system.

Considerations such as these prompted us to investigate a more homogenous system, namely the cervical sympathetic nerve trunk, where block of conduction can be ascribed only to effects on axons. Results of our observations are sum-

marized in figure 8, employing a relative concentration scale in which unity represents the concentration required to reduce the height of the conducted action potential of the B fibers in this nerve to one-half the control height. Part A of the figure shows that the functionally significant range of concentrations lies between one-half and two on this scale. Within this range, or at least in its lower half, no significant retardation of oxygen uptake was caused by chloretone, urethane, or octyl alcohol, and the effects of chloroform were small. Among the anesthetics tested, only pentobarbital caused consistent and sizable slowing of oxygen consumption throughout the relevant concentration range. As in observations on ganglia, cyanide was conspicuous for its profound oxidative effect, when compared with anesthetics.

From these observations we conclude that some, although not all, anesthetics block conduction along nerve fibers without slowing the resting oxygen consumption. It is thus clearly possible for an anesthetic to impede function in a neuronal element without significantly altering the resting rate of oxygen uptake. This evidence, on a simpler structure, is in agreement with our findings on ganglia.

ANESTHETICS AT SURGICAL CONCENTRATION

It is the main object of this paper to compare effects on function and on oxygen consumption under conditions which, for analytical purposes, are well controlled but artificial. Despite this artificiality the functional properties of ganglionic neurones retain a sensitivity to anesthetics, or at least to ether and chloroform, which is not very different from their sensitivity when normally situated in the body (Larrabee and Holaday, '52). In view of this evidence that properties of sympathetic neurones are not radically altered by excision, indications of metabolic effects to be expected in vivo may be obtained from observations in vitro. For this purpose we have in figure 9 replotted our oxygen measurements against a relative concentration scale which differs from those employed above: unity here represents approximately the

concentration of each agent required in the blood of intact animals and man to produce surgical anesthesia. It can be seen that no anesthetic depressed resting oxygen consumption of nerve or of ganglion when present in surgical concentration. Only at higher concentrations was there evidence

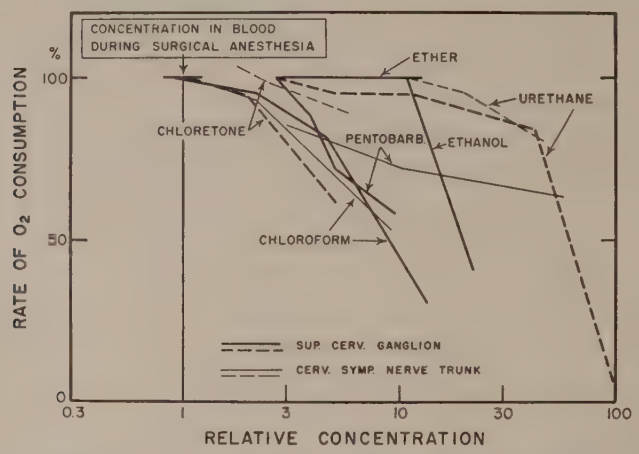

Fig. 9 Concentrations of anesthetics affecting oxygen consumption of sympathetic ganglia and nerves, in relation to concentrations required for surgical anesthesia. The concentrations required for anesthesia are given in table 1 in the introduction to this paper. These have been directly determined by analysis of blood for ether, ethanol, chloroform, and pentobarbital. Only the doses per kilogram of body weight are known for chloretone and for urethane: hence the blood concentrations of these are not accurately known and the corresponding lines are dashed in the graph.

of metabolic disturbances. This suggests that oxidative metabolism of sympathetic neurons is unaltered during surgical anesthesia.

Since our evidence on this point is indirect, and since it is a digression from the primary topic of this paper, it will not be discussed further.

OXYGEN CONSUMPTION OF ACTIVE GANGLIA

When neurons are caused to discharge and to conduct impulses, their rate of metabolism normally is increased. This has been repeatedly demonstrated by measurements of

oxygen consumption and of heat production. There is some evidence that the increment in metabolism is not due simply to an acceleration of metabolic events occurring at rest, but instead a different set of metabolic pathways is opened, or at least the various reactions which take place at rest are not all equally accelerated. Thus the "activity increment" in metabolism can be affected by certain agents in concentrations lower than those which modify the resting metabolism. For example, the activity increment in rate of oxygen consumption of frog nerve can be completely prevented by 0.1 mM sodium azide, without altering either the resting rate of oxygen consumption or the ability of the nerve to conduct impulses (Bronk, Brink et al., '48; Brink, '51).

We have, therefore, investigated the effects of three anesthetics on sympathetic ganglia during repetitive stimulation of the preganglionic nerve at frequencies approximating 10 per second; this stimulation caused the rate of oxygen consumption to rise 20 to 40% above the resting level. Oxygen uptake was measured during the second minute after starting repetitive stimulation. At this time the increase in rate of oxygen uptake exceeds 70% of the maximum increase attained after more prolonged stimulation (Larrabee and Bronk, '52). Mammalian sympathetic ganglia thus contrast with frog nerves, which change their rate of oxygen consumption much more slowly, even at comparable temperatures (Connelly, '51).

The results of applying anesthetics during activity were very different from those on resting ganglia. In the first place, oxygen consumption by active ganglia was retarded by concentrations of anesthetics considerably lower than those at which oxidative effects were found in resting ganglia (fig. 10). In the second place, metabolic changes were correlated closely with modifications in capacity to transmit impulses repetitively. In fact the increment in oxygen consumption produced by activity was always reduced in direct proportion to the depression of the postsynaptic action potential (fig. 11).

This parallelism between an oxidative and a functional effect may possibly be interpreted as support for the hypothesis that anesthetics impair function by restricting oxygen uptake, provided we assume that the primary effect is on the activity component of metabolism rather than on the resting component. This is an important possibility, since

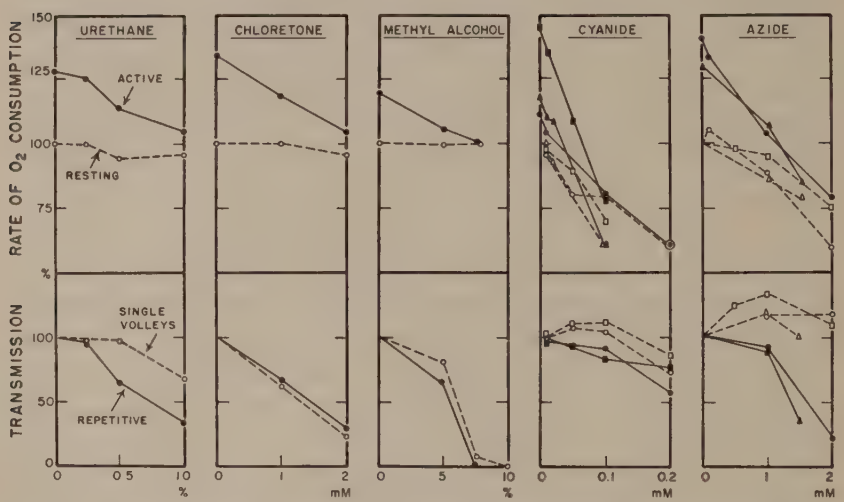

Fig. 10 Comparisons of effects of various substances on resting and on active ganglia. Activity was caused by supramaximal stimulation of the preganglionic nerve about 10 times per second. All rates of oxygen consumption are expressed as per cent of resting rate in absence of experimental agent.

Stimulation was started at the same time that the flow of bathing fluid was stopped. One minute was therefore allowed for oxygen uptake to reach a new rate before making the measurement, as a result of procedures described under "Methods." Different experiments with a particular substance are represented by differently-shaped symbols. Other conventions as indicated for urethane at left.

modification of the activity metabolism of neurons could obviously have important functional effects in the intact organism. For example, the cerebral cortex in which anesthesia is generally supposed to arise is notable for its continual, "spontaneous" activity. Therefore anesthetics might produce unconsciousness by impeding the metabolism required to support this continual activity. In advancing this sug-

gestion, we are, however, making the implicit assumption that activity is impossible without the increase in rate of oxygen consumption which is its normal accompaniment. Actually, the oxidative increment may not be as indispensable to neuronal activity as is generally supposed. For example, the activity increment in ganglionic oxygen uptake can be considerably depressed by suitable chemical agents

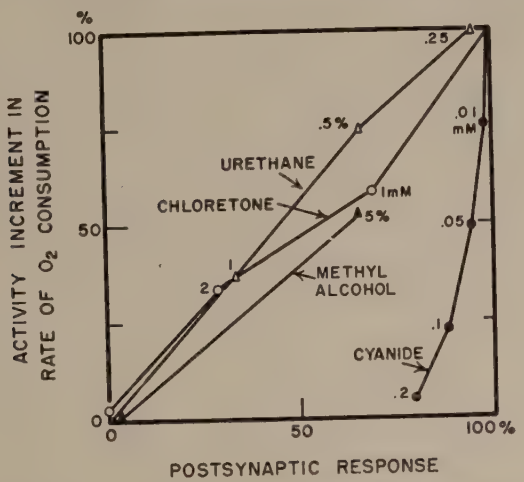

Fig. 11 Activity increment in rate of oxygen consumption (ordinate) and height of postsynaptic action potential (abscissa) in the presence of various agents during preganglionic nerve stimulation at a frequency of approximately 10 per second. Both variables are expressed in per cent of their values in the absence of experimental substances. The two variables were affected nearly equally by anesthetics; cyanide reduced the increment in oxygen uptake more than the postsynaptic response.

with relatively small impairment of the ability to transmit impulses. This is clearly illustrated by the effects of cyanide in figure 11, and by cyanide and azide in figure 10. Even more striking are experiments already cited in which azide was applied to frog nerve (Bronk, Brink et al., '48; Brink, '51). In this tissue azide can *completely* prevent the activity increment in oxygen uptake without impeding activity. These are phenomena which require further elucidation before it will be possible to understand fully the significance of the

metabolic changes which accompany activity and their modification by anesthetics.

For the reasons just discussed it is desirable to recognize that our observations on active ganglia can alternatively be explained without assuming a primarily metabolic action of an anesthetic agent. For example, an anesthetic may act through some non-metabolic mechanism, perhaps by modifying a structural component of the cellular membrane and thus impairing the capacity of neurons to discharge and to conduct nerve impulses. Depression of activity metabolism would then be a necessary consequence of the depression of activity. As indicated in the introduction to this paper, the metabolic effects of depressants on the brain in situ may be similarly explained. This has recently been discussed by Buchel and McIlwain ('50) in interpreting effects of anesthetics on phosphate metabolism of the brain.

CONCLUSIONS

Our experiments have revealed no convincing evidence that effects of anesthetics on transmission of activity along fibers and across synapses of sympathetic ganglia are due to a primary action on oxygen consumption. These observations were made on tissues, which, although excised, were otherwise intact, so that cellular activity was under experimental control. If the actions of anesthetics under these conditions are to be ascribed to slowing of oxygen uptake we must assume either (1) that the degree of slowing is quantitatively trivial, (2) that the effects on oxygen uptake are restricted to particular small portions of a neuron, such as the presynaptic terminations, or (3) that only the activity component of the oxygen uptake is affected. There seem to be no compelling reasons either for adopting or for definitively rejecting any of these three assumptions at the present time. The necessity for considering such unsupported assumptions nevertheless serves to emphasize the inconclusive nature of the experimental basis for metabolic hypotheses of anesthetic action.

It must also be recognized that measurements of oxygen utilization cannot exclude the possibility of important derangements of cellular metabolism; there is always the possibility of metabolic changes not reflected in rate of oxygen uptake. For example, suitable variations in available glucose can considerably alter the carbon dioxide production by mammalian heart muscle and by yeast cells, with little or no effect on rate of oxygen uptake (Cruickshank and Startup, '33; Aldous, Fisher and Stern, '50). For several reasons, therefore, the metabolic hypothesis of anesthetic action on neurons remains an interesting and important speculation, although at present lacking convincing experimental support.

It would obviously be unwise to attempt general conclusions concerning the central nervous system on the basis of our experiments on sympathetic ganglia, since there is ample evidence that different groups of neurons have their own characteristic properties. For example, there are at least quantitative differences between the sensitivities of various regions of the nervous system to anoxia and to narcotics. We hope, however, that our experiments will help to clarify the general problem of anesthetic mechanisms by identifying some of the variables and indicating some of the controls that must be considered for definitive investigations on neurons other than those in sympathetic ganglia.

SUMMARY

These experiments were undertaken because critical review of the literature revealed no unequivocal evidence to support the frequently advanced hypothesis that anesthetics modify functional properties of neurons by interfering with their metabolism.

Effects of anesthetics on neuronal metabolism and on synaptic transmission were compared by simultaneous measurements of oxygen consumption and of transmission in superior cervical sympathetic ganglia excised from rabbits. The excised ganglia were placed in flowing solution in a small

chamber in which oxygen concentration was measured with a polarized platinum electrode. Rate of oxygen consumption was determined by measuring the rate of fall of oxygen concentration when the flow of solution was stopped. Transmission was measured by the height of the postganglionic action potential evoked by supramaximal preganglionic nerve stimulation.

Chloretone, pentobarbital, ether, chloroform, and ethyl, amyl, and n-octyl alcohols in certain concentrations depressed synaptic transmission reversibly without slowing the resting oxygen consumption. Urethane reversibly slowed resting oxygen consumption by a small amount which did not increase significantly as the concentration rose from levels sparing to those blocking transmission. All anesthetics slowed resting oxygen consumption considerably at sufficient concentrations. In contrast with anesthetics, azide and cyanide reduced the resting rate of oxygen consumption considerably in concentrations lower than those required to depress transmission.

The extra oxygen consumption caused by repetitive activity was reduced by anesthetics in direct proportion to the depression of activity. Cyanide and certain concentrations of ozide reduced the extra oxygen consumption of activity more than the activity itself.

Similar observations were made on cervical sympathetic nerve trunks excised from rabbits. Chloretone, chloroform, urethane and octyl alcohol blocked conduction along the fibers of these nerves in concentrations lower than those which significantly retarded resting oxygen consumption. Pentobarbital was the only anesthetic found to slow resting oxygen uptake significantly at all concentrations blocking conduction. Cyanide had still greater oxidative effects.

It is inferred that anesthetics at concentrations existing in the blood during surgical anesthesia do not interfere with resting oxygen uptake by sympathetic ganglion cells or by preganglionic nerve fibers.

Thus no convincing experimental support was obtained for the hypothesis that depressant effects of anesthetics on transmission of activity along fibers and across synapses of sympathetic ganglia are due to a primary action on oxygen consumption. Functional depression may possibly be related to certain special mechanisms of oxidative interference, to other metabolic disturbances which are not reflected in changes in the resting rate of oxygen consumption, or to actions not primarily metabolic.

ACKNOWLEDGMENT

The authors wish to express their indebtedness to Dr. D. W. Bronk, who offered many stimulating suggestions and criticisms.

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CONTRACTION-ELONGATION CYCLE OF LOADED SURFACE-SPREAD ACTOMYOSIN FIBERS[1]

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FIVE FIGURES

INTRODUCTION

Any biochemical system is a model of some aspect of the living cell since information derived from the study of such systems is always extrapolated to the cell, at least by implication. Such an extrapolation is relatively simple for a system in aqueous solution, but makes for considerably greater difficulty when it involves non-dissolved components; e.g., cytostructures. Here, there is involved not only the question of the biochemical characteristics of the molecular components of such a structure, but also the question of the physical state and organization of the component molecules, and the consequent properties of the molecular aggregate.

If it be assumed that some cytostructures are formed of protein molecules surface-spread at intracellular interfaces, the study of the biological properties of protein in such a physical state can be justified (Hayashi, '51). The initial (Mazia, Hayashi and Yudowitch, '47) and subsequent investigations of this laboratory (Hayashi and Edison, '50; Kaplan, '52) gave encouragement to the assumption and led to the study of actomyosin (Hayashi, '52). This muscle protein, in the form of surface-spread fibers, was demonstrated to have the most general characteristic of muscle, that of transforming chemical energy into mechanical work.

[1] A preliminary report of this work was presented in Federation Proceedings *10:* 61, 1951.

Such investigations of the properties of an inanimate protein system must of necessity run parallel to studies of living muscle and comparisons made with the animate system. The most significant comparisons, we believe, are those which establish the most general and qualitative aspects of muscle action as existing in the model. In the present study it has been found that surface-spread actomyosin threads are capable of loaded contractions and elongations in a repeated cycle.

MATERIALS AND METHODS

The nature and preparation of the materials used have been given in detail in an earlier paper (Hayashi, '52). The various techniques of experimentation are also similar to the previous work, but with several minor modifications which should be noted.

The fibers were formed in the usual manner in the Langmuir trough, but in all cases the actomyosin solution was spread from a glass slide, and allowed to spread for at least 5 minutes. Veronal buffer was used throughout the course of the experiments, all pH values being checked with a glass electrode. Special procedures for particular experiments will be described in the following section.

EXPERIMENTS AND RESULTS

In 1941, Szent-Györgyi ('41) reported that the precipitated Weber-type (Weber, '34) actomyosin fiber, in unloaded, isodimensional, ATP-induced contraction, would return to its original dimensions if treated with a solution of KCl in high concentration, and that this fiber would then re-contract with ATP. This effect was therefore re-investigated in the loaded contractions of the surface-spread protein fibers.

The fiber, with a load tied to one end, is hung from the stationary hook, and while hanging freely in buffered (pH 7.6) 0.05 M KCl, the "resting" length is measured.[2] The

[2] The values of the weights are kept within limits such that the fiber is not stretched when the load hangs freely.

weight is then supported by means of a platform, the ATP solution (0.3% ATP in 0.05 M KCl, pH 7.6) substituted, the platform removed, and contraction allowed to proceed, the decreasing length being measured periodically. The contraction may be terminated by re-substituting the 0.05 M KCl solution, and the free-hanging shortened length measured. If now a solution of 0.3 M KCl (pH 7.6) is substituted and

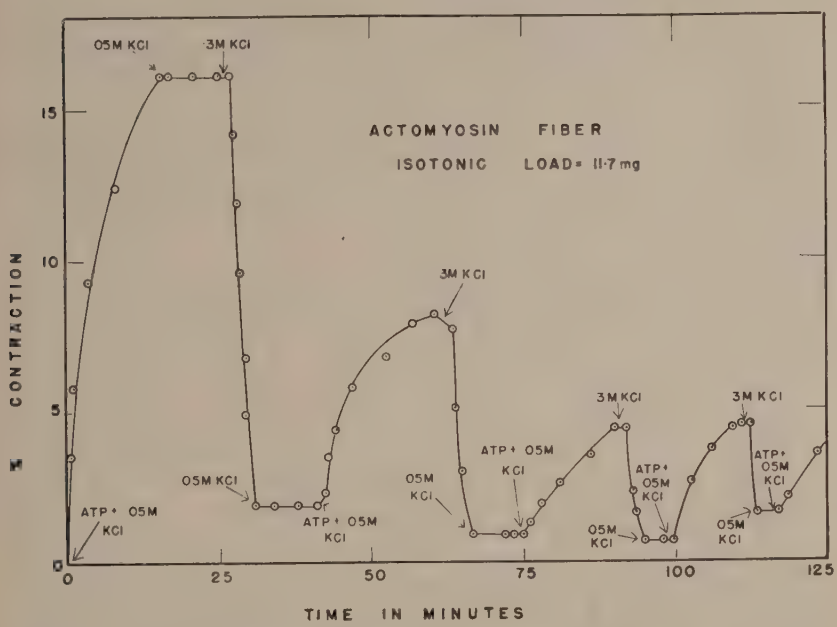

Fig. 1 Contraction-elongation cycle of fiber. Loaded, isotonic contraction and elongation of a single fiber. Solutions: 0.3% ATP in veronal buffer pH 7.6, KCl in veronal buffer pH 7.6.

the weight and fiber allowed to hang freely, the fiber will rapidly elongate (fig. 1). The elongation may be terminated also by 0.05 M KCl, and recontraction obtained if the ATP solution is subsequently applied.

The results confirm Szent-Györgyi's observations, but with loaded rather than unloaded contractions. Three points may be noted here. First, the dilute KCl seems to "fix" the fiber at any length; second, an increase in salt concentration is

necessary for elongation; and third, the presence of ATP is required for contraction.

These points are given further confirmation if the same experiment is performed, but with ATP in the same concentration added to the 0.3 M KCl solution (fig. 2). It may be seen that the fiber elongates readily in this solution also, showing that it is the shift in salt concentration responsible

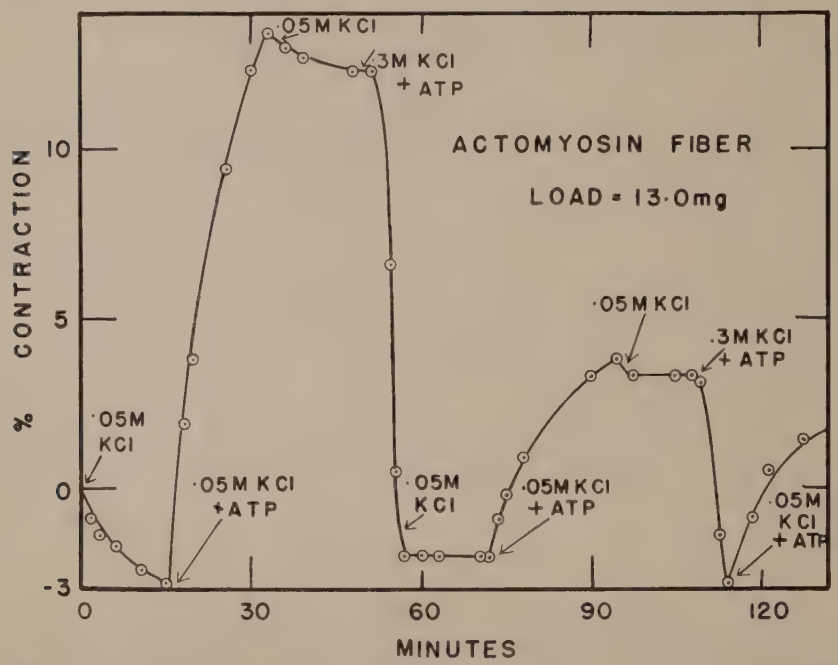

Fig. 2 Elongation of fiber in presence of ATP. Same as figure 1, but with ATP present during elongation.

for the phenomenon. The experiment shows that the condition for contraction is ATP in low salt concentration; for elongation high salt concentration.

These results throw some light on the "plasticizing" effect of high concentrations of ATP noted for precipitated fibers (Mommaerts, '50) and for glycerin-extracted and water-extracted muscle (Bozler, '51). Since ATP exists as a free acid, a considerable amount of Na or K ions must be added

to obtain a solution of ATP at a neutral or slightly alkaline pH. Therefore it is possible that the "plasticizing" effect is due to the increased ionic concentration accompanying the increase in ATP concentration.

The effects of the shift in KCl may be duplicated with the glycerinated psoas (Szent-Györgi, '49) preparation, but with some difficulty due possibly to the complicating presence of

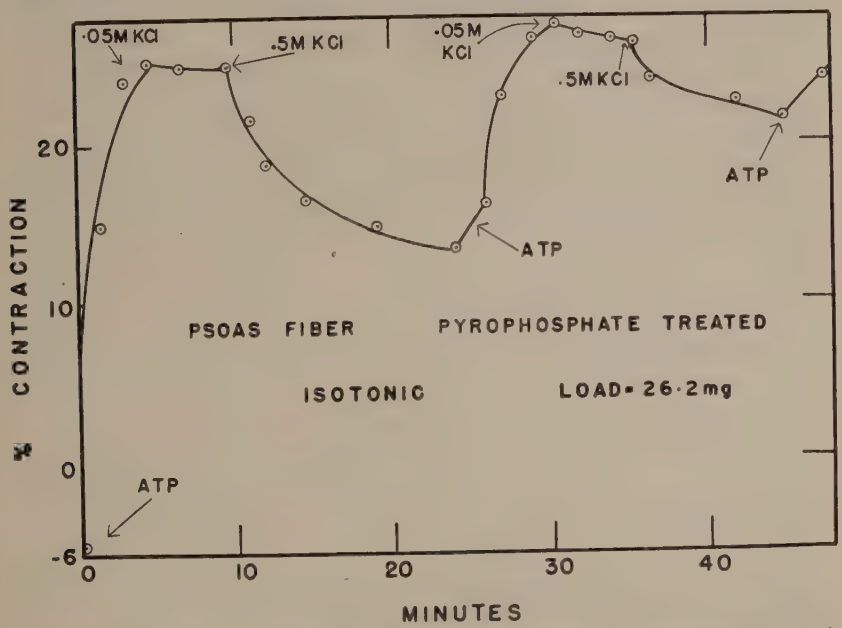

Fig. 3 Contraction-elongation in glycerinated psoas. Fibers pre-treated in 0.05 M Na-pyrophosphate + 0.05 M Na-bicarbonate, pH 8.05. Loaded, isotonic contractions.

non-contractile structural elements, or to the effect of prolonged immersion in glycerin solution of these fibers (Bozler, '51; A. G. Szent-Györgyi, '50). A. G. Szent-Györgyi has shown that such preparations, treated for a limited time with pyrophosphate, undergo a marked change in properties, with a decreased tensile strength and an increased sensitivity to salt shifts.

Figure 3 depicts the effect of a similar treatment of the glycerinated psoas. The preparation, consisting of about 5

muscle fibers, was immersed in a mixture of Na-pyrophosphate and Na-bicarbonate (0.05 M Na-pyrophosphate + 0.05 M Na-bicarbonate, pH 8.05) for 8 minutes, and loaded contractions then tested as with the actomyosin threads. The results show that, although the contractions and elongations are not so marked as with the protein threads, and although higher concentrations of KCl are necessary for elongation, similar conditions of high and low salt concentration are necessary for elongation and contraction.

The decreasing amplitude of the repeated contractions of the actomyosin threads (figs. 1 and 2) indicated the possibility that something is being lost from the fibers. Since it is well-known that actomyosin has an increasing solubility in higher salt concentrations (Szent-Györgyi, '51) it was quite possible that the 0.3 M KCl "relaxing" solution was dissolving away part of the fiber. Support for this came from the observation that the fibers, if treated with the original extracting solution (Weber-Edsall solution) of high salt content, were found to dissolve. The dissolved protein could be respread, to give perfectly active fibers again.

A careful examination, therefore, of several concentrations of KCl was made as to their effect on the recontractions. It was found that with 0.25 M KCl used as the elongating medium, the deleterious effect on the second and third contractions could be minimized (fig. 4).

Although the prospect of a salt shift being the physiological basis for muscular relaxation is a tantalizing one, other possibilities must be considered. Some preliminary studies have shown that a pH shift from 7.6 to 9.0 will bring about elongation of the fibers, but the recontraction is poor. Further studies on this pH effect are planned.

Bearing in mind the limited applicability of the fibrous elongation to muscular relaxation, it is nevertheless of some interest to investigate the question of the active or passive nature of this elongation. This question in muscle physiology is a moot one, the observations and theories of Ramsey ('44) and others calling for active relaxation, whereas the meas-

urements and interpretations of Hill ('49) call for a passive mechanism. The experiments with the fibrous system were done in the following manner.

The fiber, with a medium load, is allowed to contract in the usual manner. The platform is now raised to support the weight, the "relaxing" solution of 0.3 M KCl is substituted, and the fiber without tension is permitted to remain

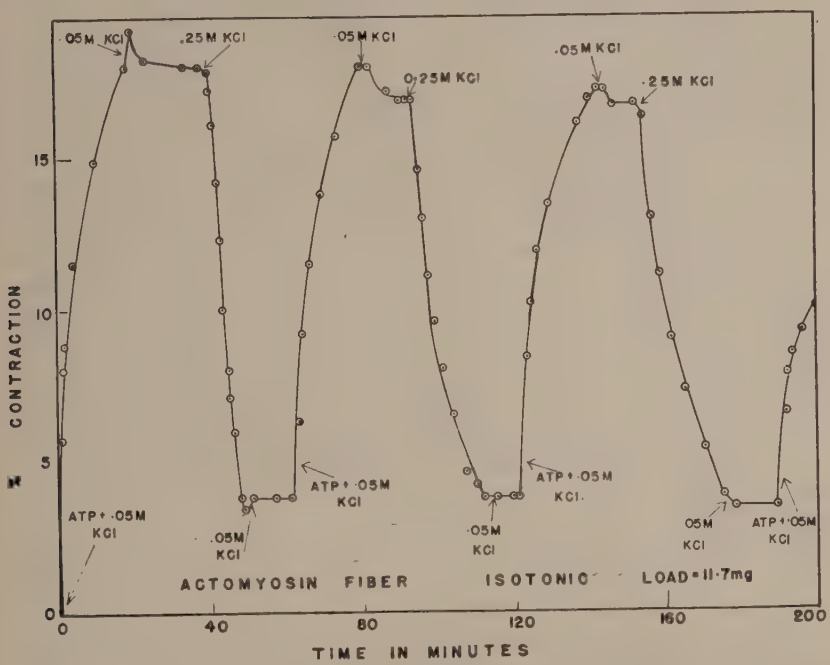

Fig. 4 Effect of 0.25 M KCl on re-contraction. To be compared to figure 1. Note that loss of activity in second and third contractions is minimized.

in this solution for some time. It follows that if elongation is an active process and not due to the work done by a free-hanging load pulling out the fiber, the fiber will lengthen without tension, the additional length resulting in increased looping or folding of the fiber. If now this new length of the fiber is "fixed" in 0.05 M KCl and the weight allowed to hang free, the increased length can be measured. This procedure was followed, with the results shown in figure 5.

In the figure, the dotted line shows the period during the first half of which the fiber is immersed in 0.3 M KCl, the latter half in 0.05 M KCl, the entire time without tension.[3] The first point at the end of this period, when the weight is hung free, is seen to give a length of fiber not appreciably

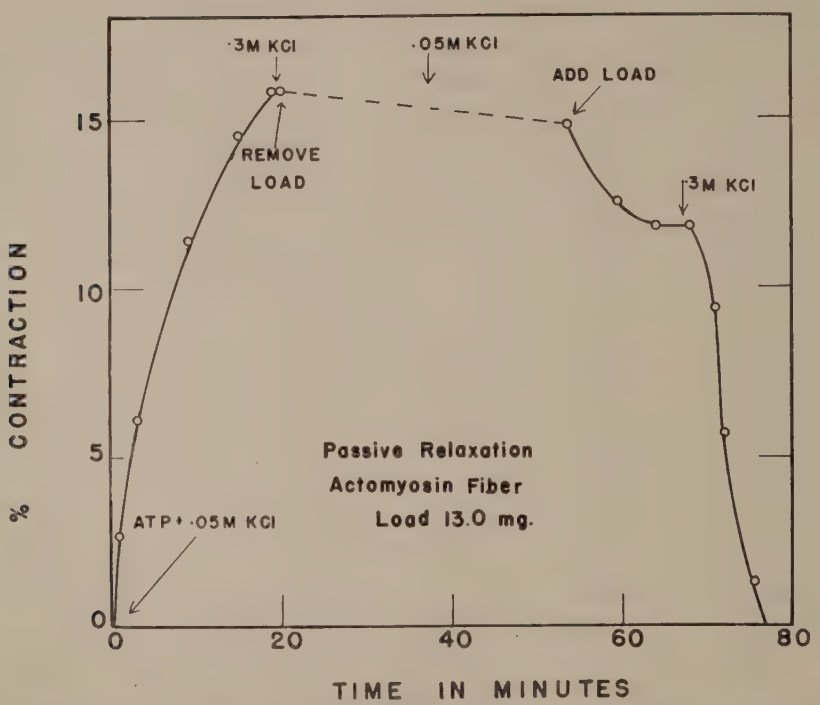

Fig. 5 Passive relaxation. Explanation in text. The first point after the interval represented by the dotted line gives full length of fiber and weight hanging freely, showing no elongation of fiber during the previous interval.

greater than at the beginning of the period. As the weight hangs free, there is a slight stretching of the fiber, which, however, becomes stabilized in the 0.05 M KCl. (This increase in length in dilute salt was not a constant feature in the experiments.) From this condition, the fiber is seen to elongate

[3] The experiment is performed in the manner described, since no measurement of length can be taken unless the weight is hanging freely.

rapidly when the higher salt concentration is applied. The results show that elongation of the surface-spread actomyosin thread in 0.3 M KCl is a passive process, not an active one.

DISCUSSION

Six years ago, Engelhardt ('46) prophetically suggested the study of surface-spread actomyosin as a key to the contractile mechanism of muscle, in all likelihood recognizing the possibility of surface-spread protein at intracellular interfaces. Fibers of surface-spread and compressed actomyosin have been studied as models of muscle in this laboratory. From the standpoint of comparison to the behavior of muscle, such models show strikingly certain qualitative characteristics possessed by muscle. Thus, they are capable of pulling a considerable load through a distance; i.e., transforming chemical energy into mechanical work, and in this capacity demonstrating that in their formation a structural continuity has been established. Moreover, the work done by the fibers is, up to a limit, determined by the load imposed on the fiber, more work being done with increasing load. It is of interest to note that these results with actomyosin have been confirmed recently by Taeschler ('52) using glycerine-extracted strips of heart muscle and give strong support to the hypothesis of Szent-Györgyi ('51) that the reaction of actomyosin with ATP is the primary reaction in the contraction of muscle.

The results of the present investigation show that the fibers possess another qualitative feature of muscle, the ability to contract and elongate repeatedly (figs. 1, 2, 4). The condition for shortening is low KCl concentration and ATP, that for elongation high KCl with or without ATP. The fact that the glycerin-extracted, pyrophosphate-treated psoas behaves, within limits, in a similar (fig. 3) fashion seems to show that the same factors operate in a system closer to intact muscle than the actomyosin threads. Bozler ('51) has obtained similar results with glycerin- and water-extracted muscle.

Low KCl concentration without ATP enables the fiber to support the weight, within limits, at any length between the

shortened and elongated condition. That is, with no ATP, the shortened condition can be maintained without loss of tension. This fact may be of some significance in relation to the "catch mechanism" of certain invertebrate muscle.

The results of the experiment depicted in figure 5 show that elongation in the fiber is a passive process; that is, the fiber elongates as a result of being pulled out by the weight. This is also in agreement with Hill ('49a) who has demonstrated in living muscle that the "heat of relaxation" is the degradation of the work done by the load in pulling out the muscle, and that living muscle does not elongate in the unloaded condition. While it is recognized that there is a difference of opinion on the question of the active or passive nature of muscle relaxation, it may be indicated that the fibers studied here are uncomplicated by the presence of noncontractile elastic elements, a factor which Hill ('49) found necessary to remove experimentally from consideration. It must be concluded that in elongation, the actomyosin fiber behaves in a passive manner in agreement with Hill's conclusions concerning the contractile elements of muscle.

Since these are isotonic contractions, the tension on the fiber is constant, and we may describe the shortening and elongation in terms of a change in the elastic modulus of the fiber. That is, in shortening and pulling the load, the fiber would be hard and elastic as compared to the elongating condition where the weight drags out the fiber to its elongated length. This would seem to agree with Hill's ('50) statements on muscle where he finds active muscle to be hard and elastic and relaxed muscle to be soft and plastic.

The nature of the forces bringing about the contraction are as yet unknown, but the effect of the varying salt concentrations could be explained on the basis of oppositely charged attractive forces at adjacent regions of the actomyosin micelle. Presumably these would be developed as a result of the interaction of the ATP with the actomyosin. The effect of increased salt concentration would then be to nullify these forces, thus bringing about a passive elongation. However,

such a mechanism could not apply unqualifiedly to muscle in light of the length-tension characteristics of muscle (Fenn, '45).

In all the foregoing speculation, there is no resolution of the problem of the nature of the interaction between ATP and actomyosin. The data do lead to the conclusion, however, that an interaction of ATP and the contractile mechanism is of primary importance in muscular shortening.

SUMMARY

1. Surface-spread actomyosin, compressed to form fibers, has been shown to be capable of repeated contractions and elongations.

2. The conditions for shortening are presence of ATP in low KCl concentration, that for elongation a high KCl concentration with or without the presence of ATP.

3. The ATP-induced contraction of the glycerinated psoas is also reversible with similar conditions, although higher salt concentrations are required for elongation.

4. The elongation of the actomyosin fiber can be shown to be of a passive nature.

5. The isotonic changes in length can be correlated to physical changes in the fiber which correspond well to physical changes that have been found in muscle.

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OXIDATIVE ENZYME SYSTEMS OF THE LARGE MILKWEED BUG, ONCOPELTUS FASCIATUS (DALLAS), AND THE EFFECT OF SABADILLA ON THEM [1,2]

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SIX FIGURES

INTRODUCTION

It is well known that oxidative enzymes such as succinic and malic dehydrogenases and cytochrome oxidase are present in mammalian tissues, and the mechanism of action of many of these enzymes is known. Extensive work has been done on the respiration of intact insects, as indicated by oxygen consumption and carbon dioxide output (Tischler, '35). The results of this type of work, however, do not give information as to the specific enzymes present in the tissues and, if present, their degree of activity. Relatively few reports have appeared in which insect tissues were used. Shafer ('15) added various non-metabolic substances such as methylene blue to tissue pulp of insects to detect the presence of dehydrogenases and oxidases. Graham ('46) used tissue slices and minced tissue with simple substrates.

The results of work on mammalian tissues indicate that fortified homogenates usually give a more accurate picture of the way oxidative enzymes function than do the pulps and slices used by Shafer ('15) and Graham ('46). Potter and Elvehjem ('36), Potter ('41, '46) and Schneider and Potter ('43a, b),

[1] Approved by the Director of the Wisconsin Agricultural Experimental Station.
[2] Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.
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have shown that in the case of mammalian tissues maximum activity of an enzyme is elicited only when the tissue reacts with the substrate in the presence of optimum amounts of the proper cofactors. In a reconstructed system of this kind, the enzyme is made the limiting factor so that it shows maximum activity. In the work to be reported this method was used.

The enzymes studied were succinic and malic dehydrogenases, and cytochrome oxidase of *Oncopeltus fasciatus* (Dallas). This work was done to ascertain: (a) whether these enzymes are present in the tissues of this insect, (b) if present, their degree of activity and properties, and (c) the effect of the insecticide, sabadilla, on them.

The toxic constituents of sabadilla are known collectively as veratrine. This mixture of alkaloids has been studied extensively for its pronounced effect on the nerves of both vertebrates and invertebrates. Allen and Brunn ('45) showed that *O. fasciatus* is highly susceptible to poisoning by sabadilla, and the gross symptoms indicate that it acts as a nerve poison. It was thought possible, however, that the respiratory system might also be affected, since many insecticides are known to depress respiration. In view of this it was of interest to determine whether sabadilla affects the respiration of *O. fasciatus*, and also whether it affects the *in vitro* activity of certain respiratory enzymes of the insect tissues.

MATERIALS AND METHODS

The large milkweed bug, *O. fasciatus*, was used in the intact, living form for respiration studies, as indicated by oxygen consumption, and in the form of homogenates of the entire insect for the study of succinic and malic dehydrogenases and cytochrome oxidase. Nine- to 11-day-old adult insects were used in all experiments. In order to obtain consistent results it was necessary for the test insects to have clean cages and an ample supply of water and food at all times. Lack of water in particular caused very low oxygen uptakes.

Intact insects. The respiration of living insects was studied by placing them in Warburg flasks and measuring their oxygen

consumption. To avoid discrepancies due to a possible difference in oxygen uptake between the sexes, one male and one female were placed in each flask. The respiratory carbon dioxide was absorbed in 0.1 ml of 10% KOH placed in the center cup. *O. fasciatus* was a good test insect for these experiments, for after exploring the Warburg flask for a few minutes they remained relatively quiet and did not come in contact with the KOH.

The experiments with intact insects were done at 23°C. and 38°C. to determine the difference in oyxgen consumption at these temperatures, and to ascertain whether the higher temperature was lethal to this insect. The effect of sabadilla on the respiration of intact insects was also determined. These experiments were continued for 12 hours (5 to 12 times as long as were the experiments with homogenates).

The sabadilla was a mixture of the entire salts of the alkaloids from which extraneous material such as oils had been removed. It was prepared by the method of Ikawa, Dicke, Allen and Link ('45) from whole seeds *(Schoenocaulin)* obtained from Merck and Company. The insecticidal preparation consisted of a 1:100 concentration of the above preparation in Superla White Mineral Oil no. 34.[4] It was applied by means of a very small brush.

If the test animals were to be used for studying the effect of sabadilla on the oxygen uptake of intact insects, the insecticide was applied to the midline of the sterna from the prothorax to the genital segments. This dosage caused knockdown within 15 minutes. If the insects were to be used for homogenates, the sabadilla was applied to the whole ventral surface. This caused knockdown in approximately one minute.

Enzyme studies. Just prior to weighing the insects their legs, antennae and wings were removed, since these were diffi-

[4] These chemicals were obtained from the following sources: sodium succinate, Eastman Kodak Co.; sodium L-malate, Pfanstiehl Chemical Co.; glutamate, Merck and Co., Inc.; nicotinamide and ascorbic acid, Nutritional Biochemicals Corp.; Superla White Mineral Oil no. 34, Standard Oil Company of Indiana; the $CaCl_2 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$, $Na_2HPO_4 \cdot 12H_2O$, and KH_2PO_4 were analytical reagent grade chemicals.

cult to homogenize and contained only a very small part of the total living tissue of the insects. Equal numbers of males and females were homogenized in sharp-pointed, glass homogenizers with sufficient glass-distilled water to make a 5% homogenate. This homogenate, the proper substrate and cofactors were placed in conventional Warburg flasks, equilibrated for 10 minutes (6 minutes in case of malic dehydrogenase), and readings were taken at 10-minute intervals for one hour. In a few experiments readings were continued at 30-minute intervals for the next 1.5 hours.

Succinic dehydrogenase. The method used was that reported by Schneider and Potter ('43a). The substrate and cofactors consisted of 1.0 ml of 0.1 M phosphate buffer of pH 7.4; 0.3 ml of 0.5 M sodium succinate; 0.2 ml of 3×10^{-4} M cytochrome c; 0.3 ml of a mixture containing 0.004 M calcium and aluminum chlorides; 0.5 ml of 5.0% homogenate; and glass-distilled water to make a total of 3.0 ml per flask.

The amounts of cytochrome c, sodium succinate and homogenate were varied from the amounts given above to ascertain their optimum concentrations. Sufficient cytochrome c to saturate the system serves to overcome the dilution effect of homogenates and insures the proper functioning of the system (Potter, '41; Schneider and Potter, '43a).

The activity of the enzyme was determined in the presence of brilliant cresyl blue, an autoxidizable dye, which can be used to replace the cytochrome system (Weil-Malherbe, '37) so as to permit succinic dehydrogenase to function when the cytochrome oxidase of the system is inhibited.

Malic dehydrogenase. The fortified substrate was essentially that reported by Potter ('46). It consisted of 0.3 ml of 0.5 M L-malate; 0.6 ml of 0.5 M glutamate; 0.6 ml of 0.1 M nicotinamide; 0.3 ml of a 0.5% solution of DPN; 0.3 ml of 3×10^{-4} M cytochrome c; 0.4 ml of 0.2 M phosphate buffer of pH 7.4; 0.3 ml of 5.0% homogenate; and glass-distilled water to bring the volume to 3.0 ml. Glutamate serves to prevent the accumulation of oxaloacetate which inhibits malic dehydrogenase. Nicotinamide inhibits one type of enzymatic breakdown of DPN.

Cytochrome oxidase. Ascorbic acid was used as substrate (Schneider and Potter, '43a, b). The reaction mixture consisted of 0.1 ml or 0.2 ml of 5.0% homogenate; 0.5 ml of 0.2 M phosphate buffer of pH 7.4; 0.3 ml of 0.004 M aluminum chloride; 1.0 ml of 3×10^{-4} M cytochrome c; 0.3 ml of 0.114 M ascorbic acid neutralized to pH 7.0 with sodium hydroxide; and glass-distilled water to give a total volume of 3.0 ml.

Correction for the autoxidation of ascorbic acid was made by subtracting each 10-minute reading for 0.1 ml of tissue from the oxygen uptake for 0.2 ml. This difference was taken as the oxygen consumption of 0.1 ml of tissue and was used for calculating the Q_{O_2} values.

The cytochrome c was prepared from beef heart muscle by a modification of the method of Keilin and Hartree ('37). The concentration of the stock solution was determined by the method described for the Evelyn colorimeter (Umbreit, Burris and Stauffer, '49).

A homogenate of 21 gm of insects was treated by the above method to determine whether cytochrome c could be isolated from the insect tissue. This preparation, homogenates, and various fractions isolated from homogenates by differential centrifugation were examined spectrophotometrically for presence of cytochrome c.

The DPN was prepared by the method of LePage ('47). The other reagents were obtained from commercial sources.[5]

The inhibition of succinic dehydrogenase by malonate and cytochrome oxidase by cyanide, diethylstilbestrol, azide and carbon monoxide was studied.

The oxygen consumption of the various enzymes is expressed as cubic millimeter uptake per hour or as Q_{O_2} (cubic millimeter of oxygen consumed per milligram of dry tissue per hour). The Q_{O_2} values were calculated on the basis of a dry weight content of 32%. This was the average of 41 individual determinations obtained by drying 1 ml of 5% homogenate. Practically all the values obtained were close to this mean value of 32%.

[5] See footnote 4, p. 509.

RESULTS

Oxygen consumption of intact, untreated, and sabadilla-treated O. fasciatus. The average oxygen consumption for pairs of insects, one male and one female, are shown in figure

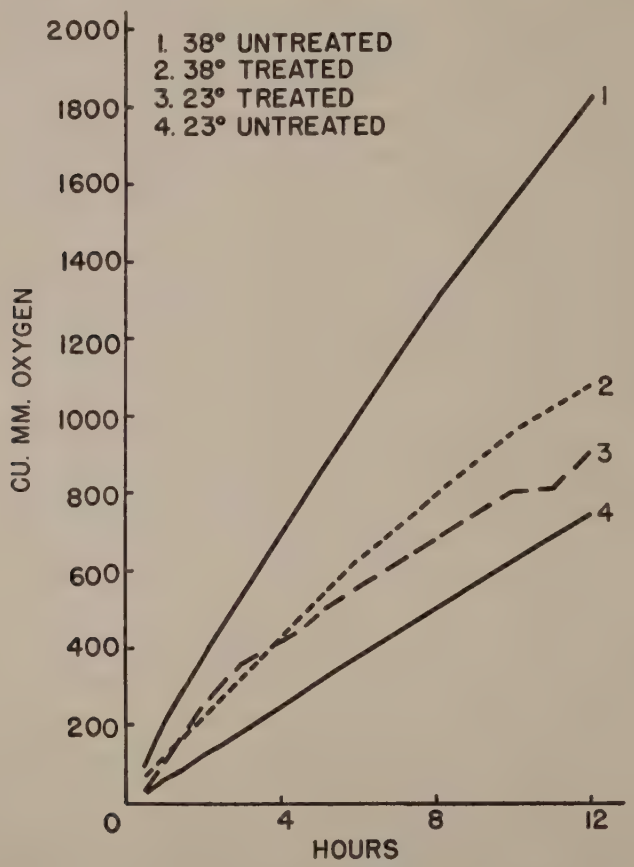

Fig. 1 Relation of oxygen consumption to time when untreated and sabadilla-treated, intact *O. fasciatus* were used in experiments at 23°C. and 38°C.

1. The values for 23°C. are based on 6, and those for 38°C. on 9 replicates. The untreated insects consumed much more oxygen at 38°C. than at 23°C., indicating a higher metabolic rate at the higher temperature. The oxygen consumption of the sabadilla-treated insects was about the same at both tempera-

tures; it was higher at 23°C., and much lower at 38°C., than for the untreated insects at the same temperatures. These results show that temperatures as high as 38°C. do not kill *O. fasciatus* in 12 hours.

Enzymes. For purposes of comparison with the above results *in vitro* studies were made of succinic and malic dehydrogenases and cytochrome oxidase of the tissues of *O. fasciatus*. The main factors considered were the effects of substrate and cytochrome c concentrations, temperature, application of sabadilla and inhibitors on the activity of these enzymes.

TABLE 1

Oxygen consumption with different amounts of tissue and succinate

TISSUE MM³ 5% HOMOGENATE	OXYGEN CONSUMPTION (MM³ FOR FIRST 30 MINUTES) SODIUM SUCCINATE (ML OF 0.5 M)[1]			
	0.15	0.3	0.6	0.9
0.25		3.9 (2)[2]	4.5 (2)	3.0 (2)
0.50	5.9 (3)	11.0 (3)	11.1 (3)	5.9 (3)

[1] Other factors same as those given in text.

[2] Figures in parentheses in this and the following tables, regardless of their position, indicate the number of runs on which the average values are based.

Succinic dehydrogenase. The amounts of sodium succinate required for maximum consumption of oxygen with 0.25 ml and 0.5 ml of 5% homogenate are given in table 1. The oxygen consumption was greater for 0.5 ml and was almost proportional for the two amounts of tissue. The relation of oxygen uptake to different concentrations of cytochrome c is given in figure 3. On the basis of these results 0.5 ml of 5% homogenate, 0.3 ml of 0.5 M sodium succinate and 0.2 ml of 3×10^{-4} M cytochrome c were used in further experiments unless otherwise indicated.

A comparison was made of the oxygen consumption of homogenates of untreated and sabadilla-treated *O. fasciatus* at 23°C. and 38°C. under the following conditions: (a) sodium succinate only, (b) sodium succinate plus cytochrome c, and (c) sodium succinate plus brilliant cresyl blue. All other known factors were present. The results are given in table 2 and those

for 38°C. are shown graphically in figure 2. The oxygen consumption per hour at 23°C. was lower than that at 38°C. Succinate alone gave relatively low values. At both temperatures homogenates in the presence of succinate and cytochrome c gave a more rapid decrease of oxygen uptake than did the corresponding runs in which succinate and brilliant cresyl blue were used.

Since the succinic dehydrogenase activity of the insect homogenates was much greater at 38°C. than at 23°C., all subsequent experiments were carried out at 38°C. It is recognized

TABLE 2

Succinic dehydrogenase activity of homogenates of O. fasciatus at 23°C. and 38°C.

SUBSTRATE AND COFACTORS	MM^3 OF O_2 CONSUMED PER HOUR			
	23°C.		38°C.	
	Untreated	Sabadilla-treated	Untreated	Sabadilla-treated
Succinate (0.3 ml of 0.5 M) without cytochrome c	0.7 (3)	1.9 (3)	6.3 (9)	6.9 (9)
Succinate plus cytochrome c (2×10^{-5} M)	1.7 (3)	2.2 (3)	19.7 (8)	32.2 (7)
Succinate plus brilliant cresyl blue (0.5 ml of 5%)	12.4 (3)	12.3 (3)	31.6 (6)	35.7 (5)

that this is a higher temperature than these insects would normally encounter for any length of time, but the results of other experiments (see fig. 1) show that 38°C. is not lethal to living *O. fasciatus* for periods much longer than the duration of these experiments.

The effects of succinate and cytochrome c on the oxygen consumption of homogenates of untreated and treated insects are given in table 3. The endogenous respiration was practically zero when cytochrome c and succinate, and succinate only, were omitted from the system. Succinate without cytochrome c gave a low but definite oxygen uptake which was essentially

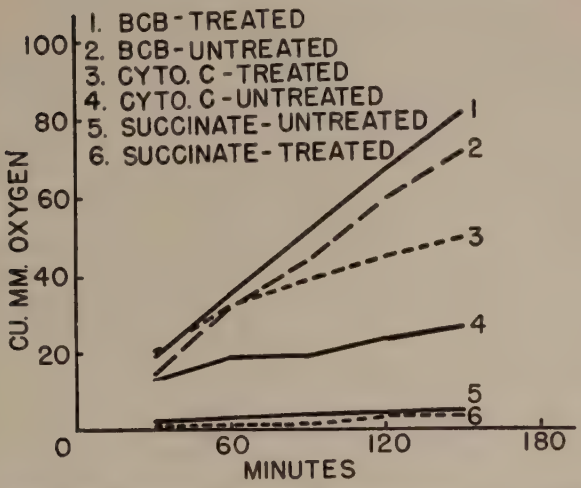

Fig. 2 Succinic dehydrogenase activity of homogenates of *O. fasciatus* at 38°C. as affected by cytochrome c (CYTO. c), and brilliant cresyl blue (BCB). Amounts and concentration of components are the same as given in table 2.

TABLE 3

Succinic dehydrogenase activity of homogenates of O. fasciatus as related to substrate and cytochrome c

SUBSTRATE AND COFACTORS	TYPE OF HOMOGENATE	NUMBER OF RUNS	OXYGEN CONSUMED PER HOUR
			mm^3
No succinate No cytochrome c	Untreated control	2	0.0
	Sabadilla-treated	2	0.0
Cytochrome c (0.2 ml 3×10^{-4} M) No succinate	Untreated control	5	0.0
	Sabadilla-treated	5	0.2
Succinate (0.3 ml of 0.5 M) No cytochrome c	Untreated control	9	6.3
	Sabadilla-treated	9	6.9
Complete system	Untreated control	25	28.1
	Sabadilla-treated	19	49.6

the same for homogenates of both untreated and treated insects. The complete system gave a much greater oxygen consumption for homogenates of treated insects, as compared with those of untreated insects. This raised the question as to whether the cytochrome c requirements for maximum activity of the two kinds of homogenates might be different. In order to obtain information concerning this possibility, a series of runs were made using different concentrations of cytochrome c.

The results of these experiments are expressed as Q_{O_2} for each 10-minute period for one hour (table 4). The standard errors of the mean Q_{O_2} values show the variation encountered in making the measurements. The oxygen consumption gradually decreased during the first hour. The consumption for one hour at the different concentrations of cytochrome c are shown in figure 3. The lowest concentration of cytochrome c, 2×10^{-5} M, gave a mean value for oxygen consumption for untreated homogenates which, statistically, was significantly less than the mean value for treated homogenates ($p < 0.05$ as indicated by t test). This difference is not present at higher concentrations of cytochrome (fig. 3). These results show that the succinoxidase system (succinic dehydrogenase plus the cytochrome system) of homogenates of *O. fasciatus* was activated by sabadilla in the presence of low concentrations of cytochrome c (2×10^{-5} M).

In view of the above results it was of interest to determine whether cytochrome c, or a similar substance, was present in the tissues of *O. fasciatus* and, if present, whether it would function more effectively in the succinoxidase system of homogenates of this insect than mammalian cytochrome c. An attempt was made to prepare cytochrome c from this insect by the method used for preparing it from beef heart muscle. The final preparation was colorless and did not contain cytochrome c as shown by failure of the reduced preparation to give increased absorption at 550 mμ when tested in a Beckman spectrophotometer. Furthermore, there was no oxygen consumption when this preparation was used in the succinoxidase system of this insect and rat liver homogenate.

TABLE 4

Succinic dehydrogenase activity of homogenates of untreated and sabadilla-treated O. fasciatus in the presence of different concentrations of cytochrome c

CONSECUTIVE 10-MINUTE PERIODS	CYTOCHROME C FINAL MOLARITY ($\times 10^{-5}$)							
	2		6		10		20	
	Untreated (25)	Treated (19)	Untreated (4)	Treated (5)	Untreated (17)	Treated (15)	Untreated (4)	Treated (4)
	Q_{O_2}							
1	6.2 ± 0.6 [1]	9.2 ± 1.1 [1]	7.4 ± 0.9	6.4 ± 1.8	9.0 ± 0.8	8.6 ± 0.4	6.8 ± 1.3	8.9 ± 1.3
2	5.4 ± 0.4	8.4 ± 0.8	4.9 ± 0.9	7.1 ± 1.6	7.4 ± 0.7	7.1 ± 0.5	7.8 ± 1.9	9.1 ± 0.5
3	3.8 ± 0.5	6.9 ± 0.7	3.9 ± 0.3	6.2 ± 1.7	4.9 ± 0.5	6.1 ± 0.5	5.4 ± 1.1	6.8 ± 0.9
4	2.6 ± 0.3	5.4 ± 0.6	3.2 ± 0.6	5.6 ± 1.2	4.7 ± 0.5	5.4 ± 0.4	5.5 ± 1.3	6.8 ± 0.2
5	2.2 ± 0.3	3.9 ± 0.4	1.8 ± 0.6	3.5 ± 1.3	2.8 ± 0.4	3.6 ± 1.0	4.5 ± 1.4	5.9 ± 0.6
6	1.4 ± 0.2	3.4 ± 0.4	2.3 ± 0.6	4.2 ± 2.2	2.4 ± 0.3	3.3 ± 0.6	5.0 ± 1.3	5.9 ± 0.3
Av.	3.6	6.2	3.9	5.5	5.2	5.7	5.8	7.2
Cubic centimeter oxygen uptake per hr.	28.1	49.6	31.3	44.0	41.6	45.5	46.7	57.9

[1] Standard error of the mean.

Homogenates of whole insects were centrifuged at 18,000 g., and the supernatant fractions were reduced by sodium hydrosulfite and tested spectrophotometrically. Cytochrome c was not detected. Fractions isolated from sucrose homogenates by differential centrifugation also failed to show increased absorption, and cytochrome bands were not detected by observing whole homogenates with a hand spectroscope. These results

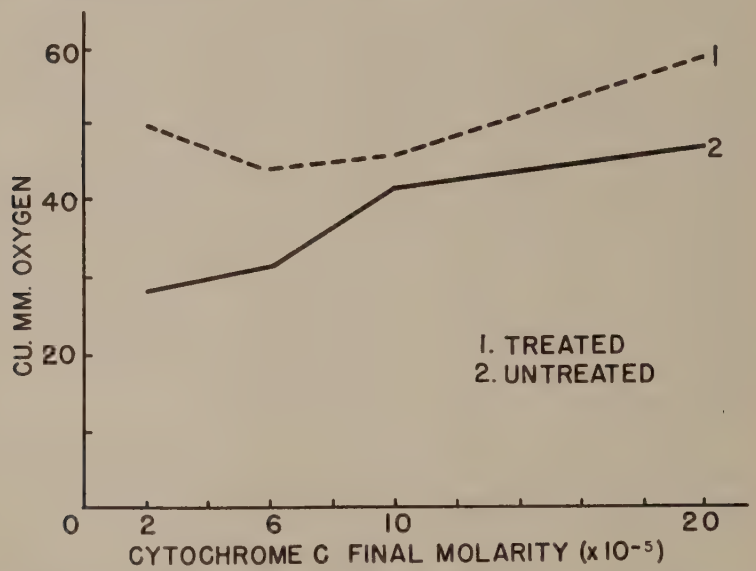

Fig. 3 Relation of different concentrations of cytochrome c to the succinic dehydrogenase activity of homogenates of *O. fasciatus*. The activity is given in terms of oxygen consumption per hour.

show that either cytochrome c as found in mammalian tissues is not present in *O. fasciatus*, or is present in too minute quantities to furnish anywhere near the concentrations used in these experiments.

Sabadilla added directly to the flasks inhibited oxygen consumption of homogenates of untreated insects, but the oxygen uptake of homogenates of sabadilla-treated insects was greater than that of the untreated insects. The oil used for suspending the sabadilla, when placed on the intact insects in the same way

as the sabadilla-oil mixture, did not affect the oxygen consumption (table 5).

The data in table 6 show that malonate, diethylstilbestrol, cyanide, and azide, respectively, inhibited the succinoxidase system of homogenates of *O. fasciatus*. Malonate acts directly on succinic dehydrogenase (Quastel and Wooldridge, '28), and

TABLE 5

Effect of sabadilla and oil on the succinic dehydrogenase activity of homogenates of O. fasciatus in vivo and in vitro

TYPE OF HOMOGENATE	NUMBER OF RUNS	OXYGEN CONSUMED PER HOUR
		mm^3
Untreated (sabadilla added to flask)[1]	5	31.3
Untreated (no sabadilla added)	5	42.9
Sabadilla-treated control[2]	3	85.6
Oil treatment[3]	5	55.1
Untreated (no oil or sabadilla)	5	55.6
Sabadilla-treated control[2]	3	85.6

[1] Saturated aqueous solution of sabadilla placed directly in the flask with untreated homogenate.

[2] Live insects treated with sabadilla in oil prior to homogenizing.

[3] Oil used as carrier for the sabadilla was applied to the living insects in the same way the sabadilla in oil was used in all other experiments.

TABLE 6

Inhibition of succinoxidase system of homogenates of O. fasciatus

INHIBITOR	FINAL MOLARITY OF INHIBITOR	FINAL MOLARITY CYTOCHROME C ($\times 10^{-6}$)	MM^3 O_2 CONSUMED PER HR.	
			Untreated (2)	Sabadilla-treated (2)
Control	..	2	37.7	47.6
Sodium malonate	0.007	2	3.4	7.2
Diethylstilbestrol	0.0001	2	9.9	9.6
Sodium azide	0.003	2	17.9	36.9
Control	..	10	31.3	56.4
KCN	0.001	10	3.7	9.9
KCN + brilliant cresyl blue (0.5 ml 0.5%)	0.001	10	44.7	55.3

its overall effect is shown in figure 4. Diethylstilbestrol (McShan and Meyer, '46), cyanide, and azide inhibit the cytochrome oxidase of this system. The effect of diethylstilbestrol is shown in figure 5.

The oxygen consumption of the insect homogenates was not prevented by cyanide in the presence of brilliant cresyl blue.

Malic dehydrogenase. The tissues of *O. fasciatus* contain an enzyme which activates malate when the same conditions are used as for the study of this enzyme in mammalian tissues.

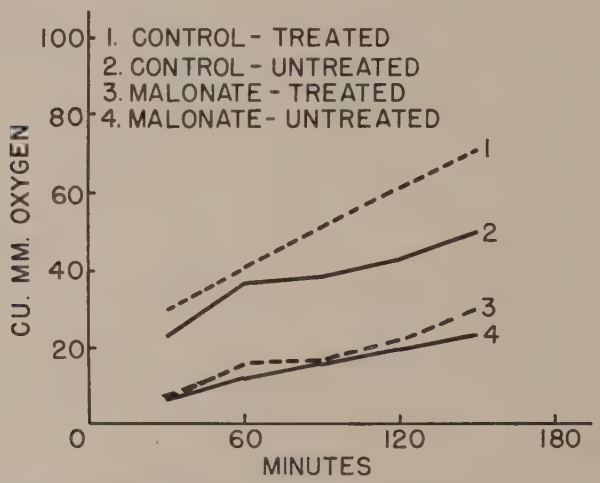

Fig. 4 Malonate inhibition of succinic dehydrogenase of homogenates of *O. fasciatus*.

The homogenates of untreated and sabadilla-treated insects consumed essentially the same amount of oxygen per hour. The oxygen uptake of both kinds of homogenates was inhibited by diethylstilbestrol, which is known to inhibit the cytochrome oxidase of the malate system (table 7). The activity of malic dehydrogenase decreased at a greater rate than did the activity of succinic dehydrogenase.

Cytochrome oxidase. The cytochrome oxidase activity of untreated and sabadilla-treated homogenates of *O. fasciatus* is given in table 8 in terms of the mean Q_{O_2} values for the first two 10-minute periods. The cubic millimeters of oxygen consumed

in 40 minutes is plotted against time in figure 6. The oxygen uptakes varied considerably for runs made on different days, but different runs made on the same day checked closely. The homogenates of sabadilla-treated insects gave consistently

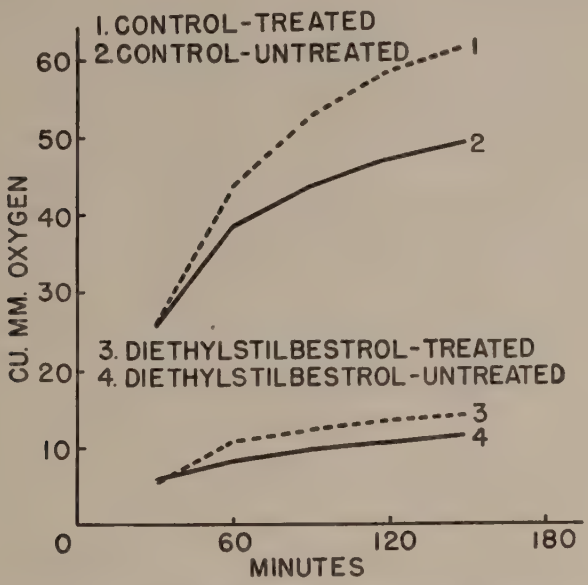

Fig. 5 Diethylstilbestrol inhibition of the succinoxidase system of homogenates of *O. fasciatus.*

TABLE 7

Inhibition of the malic dehydrogenase system of O. fasciatus by diethylstilbestrol

INHIBITOR	FINAL MOLARITY OF INHIBITOR	MM^3 O_2 CONSUMED PER HOUR	
		Untreated	Sabadilla-treated
Control	..	21.1 (7)	23.5 (7)
Diethyl-stilbestrol	0.0001	5.4 (3)	8.5 (3)

higher oxygen uptakes and, as shown in table 8, the averages for the treated were almost 100% greater than those for the untreated homogenates. The activity of the oxidase was much greater than that of the succinoxidase. Treated homogenates of the former had a Q_{O_2} of 133.0, as compared with 9.2 for the

latter (tables 8 and 4). The oxygen consumption declined rapidly during the first hour. The oxidase was completely inactivated by boiling.

Cytochrome oxidase when functioning as part of the succinoxidase system was inhibited by diethylstilbestrol, cyanide,

TABLE 8

Cytochrome oxidase activity of homogenates of O. fasciatus

CONSECUTIVE 10 MINUTE PERIODS	AVERAGE Q_{O_2} (6)	
	Untreated	Sabadilla-treated
1	84.3 ± 16.2 [1]	133.0 ± 31.3 [1]
2	56.4 ± 16.4	105.1 ± 23.8

[1] Standard error of mean.

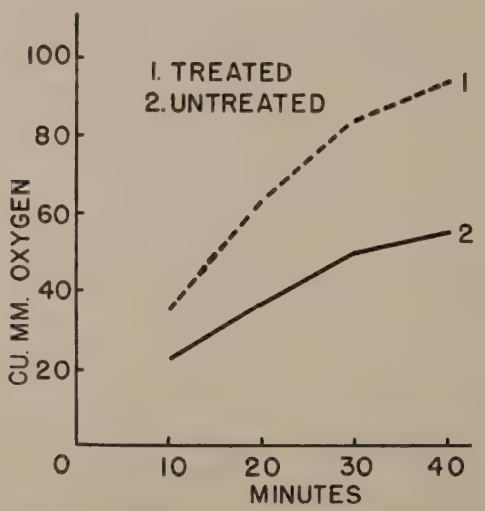

Fig. 6 Cytochrome oxidase activity of homogenates of *O. fasciatus.*

and azide (table 6). Carbon monoxide also inhibited the oxidase, but on the basis of present evidence it appears that this inhibition was not reversed by light.

Discussion. The results presented in figure 1 show that an increase in temperature from 23°C. to 38°C. caused an increase in the metabolic rate of untreated living *O. fasciatus.* The insecticide sabadilla stimulated respiration at 23°C. and de-

creased it at 38°C. The oyxgen consumption of treated insects was approximately the same at the two temperatures and was intermediate to the values found for the untreated insects. These results indicate that the effect of sabadilla on living insects in terms of respiration was reversed with a sufficient rise in temperature although results of *in vitro* experiments show that the activity of succinoxidase and cytochrome oxidase was increased by sabadilla at 38°C. when homogenates were used.

Homogenates of *O. fasciatus* contain enzymes that activate succinate and malate, as shown by oxygen consumption, under conditions similar to those used for the study of succinic and malic dehydrogenases in mammalian tissues. The optimum concentration of cytochrome c required for the succinoxidase system of the tissues from untreated insects is greater, however, than the requirement for rat liver. The activity of these dehydrogenases is much lower than the values reported for most mammalian tissues (Potter, '46; McShan, Erway and Meyer, '48). On the other hand, the cytochrome oxidase activity of insect homogenates is approximately one-third as great as that of rat liver.

Brilliant cresyl blue, which accepts hydrogen from succinic dehydrogenase of mammalian tissues, was also found to accept it from the dehydrogenase of insect tissues (fig. 2). Succinic dehydrogenase was inhibited by malonate, and the cytochrome oxidase by cyanide, diethylstilbestrol, azide, and carbon monoxide, respectively. These compounds are known to inhibit the above enzymes of mammalian tissues in a similar manner.

Homogenates of sabadilla-treated insects showed significantly greater succinoxidase activity at 38°C. than did homogenates of untreated *O. fasciatus* in the presence of low concentrations of cytochrome c, namely, 2×10^{-5} M (fig. 3). Presumably sabadilla facilitates the oxidation of succinate by insect homogenates in the presence of small amounts of cytochrome c. This effect is due to the insecticide and not to the oil in which it is suspended, as shown by the results in table 5.

In view of the above results with the succinoxidase system, it is of interest that sabadilla did not stimulate an increase in

oxygen consumption by the malic oxidase system at the low concentration (2×10^{-5} M) of cytochrome c. The activity of this system also decreased rapidly. This can be explained on the basis of unpublished results in which it was found that DPN is rapidly broken down by an enzyme present in the tissues of this insect which uses DPN as substrate, and which is not inhibited by nicotinamide. This results in the decrease of DPN to a level that does not elicit the maximum activity of the malic system.

The cytochrome oxidase activity of homogenates of sabadilla-treated insects was much greater than that of homogenates of untreated insects (table 8). This is also true, but not to as great an extent, for succinoxidase in the presence of low concentrations of cytochrome c (fig. 3), and for living, intact insects at 23°C. (fig. 1). These stimulating effects of sabadilla may be indicative of a compensatory mechanism which the poisoned insect brings into play to combat the effect of the insecticide. Possibly in the intact insect the sabadilla acts directly on one or more enzymes; or indirectly in that the insect initiates a protective mechanism which results in increased mobilization of energy through increasing respiration. This could be accomplished by the insecticide counteracting an inhibitory mechanism that normally controls the activity of these enzymes. The latter possibility would seem to be the more likely.

It is of interest in this connection that sabadilla affects the electrical properties of nerves (Graham and Gasser, '31). On the basis of this property the insecticide may cause changes in critical potentials (for example, oxidation-reduction) which are conducive to an increase in the activities of enzymes such as succinoxidase and cytochrome oxidase. Since the effect occurs within a few minutes after applying the sabadilla, it does not seem likely that the increase in respiration is due to increased synthesis of enzymes by the tissues, either in intact insects or in homogenates.

The mechanism by which succinic dehydrogenase of homogenates of *O. fasciatus* is linked to the highly active cytochrome

oxidase is of interest, since cytochrome c was not obtained from insect tissues by extraction, or detected in homogenates and fractions of homogenates by spectrophotometric tests. These results indicate that if cytochrome c is present in the tissues of this insect, the concentration is extremely low. Similar results have been reported for *Arbacia* eggs (Ball and Meyerhof, '40, Clowes and Krahl, '40; Krahl, Keltch, Neubeck and Clowes, '41). On the basis of these results it is clear that succinic dehydrogenase of *O. fasciatus* may be linked normally to cytochrome oxidase by some factor other than mammalian cytochrome c, although the succinoxidase system of this insect's tissues is capable of using mammalian cytochrome c *in vitro* as shown by the results of experiments reported here. This theory is supported by other evidence. For example, the low respiration which occurred in the reconstructed succinoxidase system when only cytochrome c was omitted (table 3) was probably due to this factor. Furthermore, brilliant cresyl blue is more effective in transporting hydrogen from the insect dehydrogenase to oxygen than is cytochrome c. This effect was more pronounced with homogenates of sabadilla-treated insects (table 2 and fig. 2). On the other hand, this dye is less effective than cytochrome c in transporting the hydrogen of succinic dehydrogenase of mammalian tissues, such as rat liver, to oxygen (McShan and Meyer, '46).

SUMMARY

The effect of the insecticide, sabadilla, on the respiration of *Oncopeltus fasciatus* (Dallas) and on the enzymatic respiration of homogenates of this insect was studied.

Both untreated and sabadilla-treated insects lived for more than 12 hours at 38°C. Untreated insects had a higher oxygen uptake at 38°C. than at 23°C.; sabadilla-treated insects had an oxygen uptake at 23°C. that was greater than, and at 38°C. that was less than, that of untreated insects at the same temperatures.

Succinic and malic dehydrogenases and cytochrome oxidase were shown to be present in homogenates of this insect. The

succinic dehydrogenase activity of homogenates of both untreated and treated insects was higher at 38°C. than at 23°C. The activity of the cytochrome oxidase was much higher than that of the dehydrogenases. As in mammalian tissues, succinic dehydrogenase was inhibited by malonate, and cytochrome oxidase was inhibited by diethylstilbestrol, cyanide, azide, and carbon monoxide, respectively.

The requirements of cytochrome c for maximum activity of the succinoxidase system were not the same for homogenates of untreated and sabadilla-treated insects, the latter being more sensitive to low concentrations. Cytochrome c could not be isolated or detected spectrophotometrically in the tissues of this insect.

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THE ACTION OF TANNIC ACID ON THE ERYTHROCYTE MEMBRANE[1]

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SIX FIGURES

INTRODUCTION

Among the most striking of the effects produced on the erythrocyte by low concentrations of tannic acid (TA) is the great and instantaneous reduction in its characteristic permeability to anions (Handovsky and Heubner, '23; Jacobs, Stewart and Butler, '43), the movement of non-electrolytes being at the same time unaffected. Additional effects on the same cell include agglutination, changes in sedimentation rate, changes in osmotic resistance (Handovsky and Masaki, '23; Bohlmann, '44), susceptibility to phagocytosis by leucocytes of the same animal (Schade and Weiler, '27), disc to sphere transformation (Jacobs, unpublished), protection against the lytic action of soaps (Buckendahl, '33) and of saponin (Handovsky and Masaki, '23), and finally, morphological changes in the surface of the cell observed with the electron microscope (Bohlmann, '44).

The erythrocyte is especially suited for use in investigating the effect of TA on the movement of ions and non-electrolytes, since it lends itself readily to the quantitative measure-

[1] Large portions of this study formed part of a dissertation presented to the faculty of the Department of Physiology of the University of Pennsylvania School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ment of permeability. Furthermore the wealth of existing information about its surface, a large part of which has been gained by chemical or physical analysis of the isolated membrane (ghost), facilitates the interpretation of new results. Accordingly the erythrocyte has been chosen for this investigation, which principally concerns the action of tannic acid on the permeability of the cell. Aspects dealt with include the effect of varying TA concentration, the effect of varying cell concentration, the reversibility of the effect, the site of action, the complicating effects of the coincidental change in osmotic resistance, the effect on permeability to non-electrolytes and the use of TA as a research tool.

METHOD

Handovsky and Heubner ('23), by noting the change in color when hemoglobin is oxidized to methemoglobin, demonstrated that TA impedes the entrance into the erythrocyte of the nitrite ion. A more generally useful method, which can be used for following the entrance of any anion, is the photoelectric observation of osmotic volume changes when the erythrocyte is placed in an isosmotic solution of an ammonium salt (Jacobs, Stewart and Butler, '43). In the case of NH_4Cl, a salt used throughout this investigation, the rate of swelling is dependent on the rate of exchange of external Cl^- for internal OH^- (Jacobs, '27) and is as a consequence decreased by tannic acid. A suitable quantitative measure of the degree of retardation of ionic diffusion proved to be R, the ratio of the time required for any degree of swelling or hemolysis in the presence of tannic acid, T_{TA}, to the corresponding time in its absence, T_C, thus:

$$R = T_{TA}/T_C$$

Treatment of materials

In order to obtain reproducible results when working with low concentrations of TA, special precautions must be observed to prevent changes in the cell suspension and in the

TA solution. This compound has a high affinity for proteins and phospholipids as well as for clean glass and tends in consequence to be inactivated by these substances. Indeed it was found that practically all the TA in an 8×10^{-5} % solution was removed in a short time by the walls of a flask which had previously been cleaned, heated to incandescence for two minutes to remove organic material and cooled. The problem of adsorption of TA by glass or by organic films on the glass was met by using silicone[3]-coated flasks which were first equilibrated with TA solutions of an appropriate concentration. Adsorbed organic matter is easily removed from the silicone surface by scrubbing with Dreft (a Procter and Gamble detergent).

With regard to the effect of protein and phospholipid, since their presence in traces can never be completely avoided in a cell suspension, the problem is to maintain their concentrations constant in any single series of tests. A complication arises, however, since as Stewart and Jacobs ('43) observed, a washed cell suspension rapidly becomes less susceptible to TA because of the release from the cells of a neutralizing substance or substances. It has been found in the present investigation that this difficulty can apparently be surmounted if the suspension is maintained concentrated and refrigerated until shortly before its use. To avoid premature dilution and stirring, the samples of suspension are delivered dropwise from a paraffin-coated buret-like arrangement using, instead of a stop-cock, an air-leak above the column to control flow. Tests have shown that by discarding the first drop before each sample, a very constant amount of suspension and number of cells can be delivered at any desired intervals over a 45 minute period.

Further precautions include the use of paraffin-coated vessels for washing and storing the cells and the exercise of extreme gentleness in handling suspensions in order to avoid bubbles or traces of hemolysis. If $NaHCO_3$, in very low concentration, is added to the external solution, the HCO_3^- ion

[3] DC 804, Dow-Corning Corp., Midland, Michigan.

facilitates the penetration of NH_4Cl by a mechanism termed "catalyzed diffusion" (Jacobs, '40) without altering R. This procedure greatly decreases the time required to run a test and thereby minimizes bacterial action and spontaneous deterioration of the cells.

Apparatus

For obtaining the end points, an inexpensive photoelectric thyratron circuit, commonly used for furnace thermostat control, was suitably modified (fig. 1 A) to compensate for fluctuations in the light source and to allow the determination of complete hemolysis curves. As may be seen in the optical diagram (fig. 1 B), a cylinder mounted on a micrometer shaft is used as a variable obstruction in the path of light to one phototube. This determines the intensity of the light which must be transmitted by the suspension in order to discharge the thyratron circuit. A zero adjustment, casting a second shadow, is provided to compensate for small changes as the optical system heats up. For optimum operation the grid lead to the phototubes should be of minimum length.

In practice, micrometer settings for discharge at any desired degree of swelling or hemolysis are predetermined by the use of standards made up of cells and isotonic hemolysate. The test suspension is then inserted, the micrometer pre-set and the auditory signal from the relay awaited.

Procedure

In running a hemolysis test, one or more drops of a suspension of human erythrocytes, previously washed twice in buffered saline of pH 7.4 (2% M/8 Na_2HPO_4-NaH_2PO_4 by volume) containing 6×10^{-5} M $NaHCO_3$, are added to 1 ml of a similar but blood-free solution in a silicone-coated tube. Immediately thereafter, 8 ml of M/6 NH_4Cl are mixed with 1 ml of TA solution (also in M/6 NH_4Cl) in another silicone-coated tube; this solution is poured rapidly into the tube containing the suspension, and the mixture, now at pH 6.3,

is swirled gently. The manner of mixing should be identical in all cases to avoid the possibility of variation of CO_2 content with its great effect on the rate of ionic exchange. When the end point, generally 40% hemolysis, is neared, the contents are transferred to an uncoated tube for photoelectric observation. Temperature should be maintained constant. In

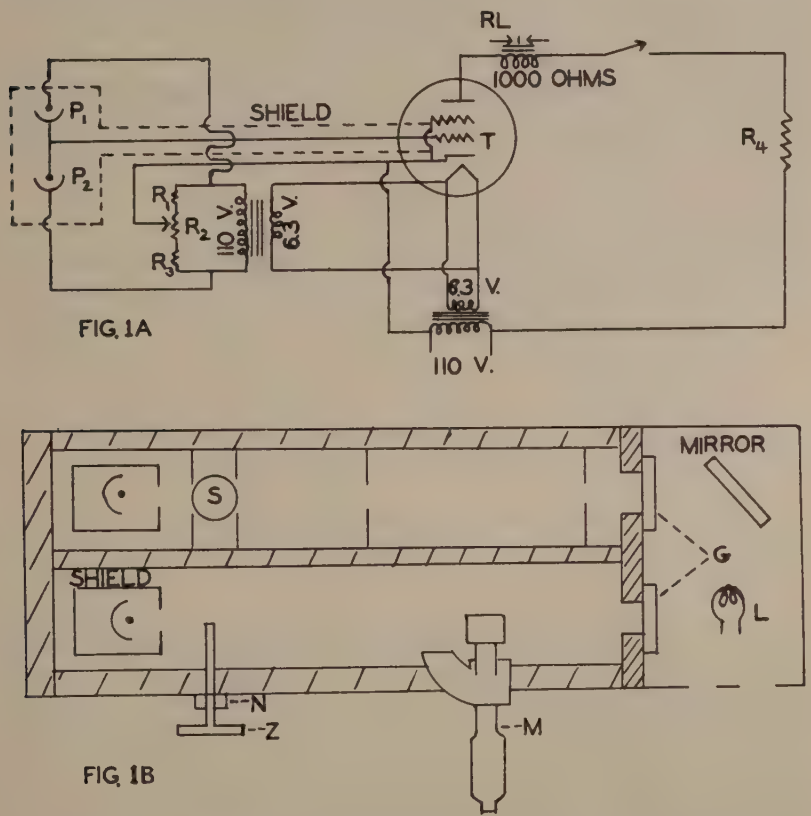

Fig. 1 Circuiting and optics of the photoelectric instrument.

A Electrical circuit: (T) 2050 gaseous tube; (P_1, P_2) 922 phototubes; (RL) relay having 12 v. closing voltage; (R_1, R_3) 50,000 ohms; (R_2) 10,000 ohms, variable; (R_4) 3000 ohms, for limiting plate current to 40 ma.; light from suspension goes to P_2.

B Optical system: (S) tube, 25 mm × 200 mm, containing the suspension; (G) glass plates for limiting heat flow; (L) 100 watt bulb, 110 v.; (Z) bolt for zero adjustment; (N) nut mounted on wall of case; (M) micrometer with part of frame removed.

all experiments, observations were made to be sure that agglutination was not occurring, this undesirable condition often appearing at TA concentrations above 0.005%.

RESULTS

Effect of varying the TA concentration

At constant cell concentration, the increase in the degree of retardation of hemolysis with increase in concentration of TA is more nearly geometrical than arithmetical as may be seen in table 1. In fact, maximal effects for a 24 hour

TABLE 1

Effect of concentration of tannic acid upon the time of hemolysis in M/6 NH_4Cl at pH 6.3. Cell concentration is held constant (1.2×10^6 cells per ml)

CONCENTRATION OF TANNIC ACID (PER CENT)	TIME FOR 40% HEMOLYSIS
0	7 min. 1 sec.
8×10^{-6}	7 min. 26 sec.
1.6×10^{-5}	8 min. 35 sec.
4.0×10^{-5}	14 min. 35 sec.
8.0×10^{-5}	79 min. 30 sec.
1.0×10^{-4}	165 min. 53 sec.
1.2×10^{-4}	344 min. 47 sec.
1.4×10^{-4}	$>$ 600 min.

experiment are obtained at relatively low TA concentrations. For example, a dilute well-washed suspension took only 7 minutes to hemolyze in M/6 NH_4Cl but in the presence of 5.5×10^{-5}% TA was still largely unhemolyzed after 20 hours.

Under conditions somewhat different from those described above in the section on procedure, the lower limits of TA concentration at which a significant degree of reduction in permeability may be obtained seem almost unbelievable. In this method the cells are washed at pH 8, no $NaHCO_3$ is added, and an end point of 80% hemolysis is used rather than one of 40%. The probable reasons for the success of this procedure will be discussed later. In one such experiment, which could be reproduced at will, with a cell suspension contain-

ing 5500 cells per cubic millimeter and a TA concentration of $2.5 \times 10^{-7}\%$ (i.e. 1.5×10^{-9} M), the time of hemolysis was prolonged by 17%.

Effect of varying the cell concentration

As might be expected, the magnitude of the effect of a given amount of TA in a given volume of solution is dependent upon the number of cells present. This effect is shown in table 2. It is seen that here, too, the relation is non-linear, the hemolysis times increasing in an almost geometrical progression as the number of cells is diminished.

TABLE 2

Effect of concentration of cells upon the time of hemolysis in M/6 NH_4Cl, pH 6.3, in the presence of tannic acid

NUMBER OF CELLS ($n = 2.7 \times 10^9$ CELLS/ml)	CONCENTRATION OF TANNIC ACID (PER CENT)	TIME FOR 40% HEMOLYSIS
n, 2n, 3n	0	6 min. 15 ± 10 sec.
n	1.8×10^{-4}	52 min. 49 sec.
2n	1.8×10^{-4}	16 min. 11 sec.
3n	1.8×10^{-4}	9 min. 41 sec.

With equal volumes of solutions and varying cell concentrations, the retardation ratio, R, can be held constant by varying the amount of TA present. When this is done it is interesting to note that there is an approximately linear relation between the concentration of TA and the number of cells present (fig. 2). This appears to indicate that effects of the same magnitude in different samples require the presence of approximately the same amount of TA per cell in excess of a certain minimum. Contrary to outward appearances, however, this graph can neither be used to determine the amount of TA taken up per cells, nor the amount of free TA left in solution, as will be shown in a later paper.

Reversibility of the TA effect

The original observation of Jacobs, Stewart and Butler ('43) that the effect of TA could be "more or less completely reversed" by washing the cells in gelatin-containing saline was investigated further. It was found that the use of gelatin as a reversing agent imposes on the cells a seeming "injury" which is greater the longer the exposure to TA before the addition of gelatin. This injury is indicated by acceleration of hemolysis in NH_4Cl. It is believed on the basis of

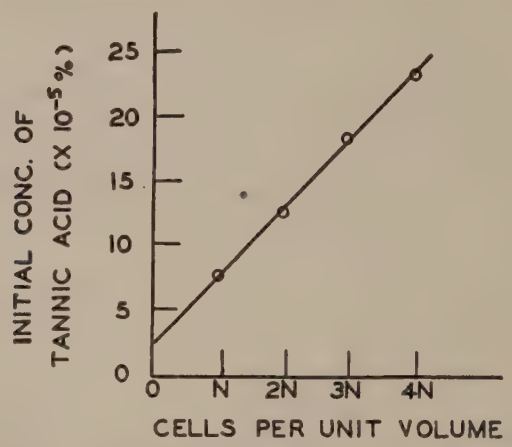

Fig. 2 Amount of tannic acid required to maintain a constant degree of retardation of ionic movement at various cell concentrations. R = 2.8. pH = 6.3.

later investigation that TA produces a decrease in osmotic resistance, which ordinarily is masked by the simultaneous decrease in permeability. Gelatin would serve to remove sufficient TA to neutralize the effect on permeability, while the decrease in osmotic resistance would remain to manifest itself in an acceleration of hemolysis. A decrease in osmotic resistance after simple exposure to TA has in fact been reported by Bohlmann ('44). In achieving reversal (table 3), 0.4 ml of 1% gelatin was added to 10 ml of washed beef cell suspension in TA-NaCl solution. After centrifuging and washing once again in 10 ml of saline (pH 6.9), all but

1 ml of the supernate was replaced with 10 ml of M/6 NH_4Cl (pH 7) and the hemolysis time measured. As can be seen in table 3, the tests were arranged in such an order that aging of the cells could be eliminated as a significant factor.

Washing alone is also effective in removing TA from treated cells, but even when an alkaline wash is applied (TA being more weakly bound at high pH) reversal reaches

TABLE 3

Reversal of the tannic acid effect on beef erythrocytes by the addition of gelatin at various times after exposure of the cells to tannic acid

CONCENTRATION OF TANNIC ACID %	TIME INTERVAL BETWEEN EXPOSURE TO TANNIC ACID AND THE ADDITION OF GELATIN	TIME AT WHICH HEMOLYSIS TEST WAS STARTED	TIME FOR 75% HEMOLYSIS
0	No gelatin added	16:30	3 min. 1 sec.
		17:45	3 min. 5 sec.
0	30 sec.	16:43	3 min. 3 sec.
		17:12	3 min. 8 sec.
0.005	No gelatin added	16:35	15 min. 24 sec.
0.005	18 sec.	16:49	3 min. 3 sec.
		17:24	3 min. 10 sec.
0.005	2 min.	17:18	3 min. 6 sec.
		17:30	2 min. 58 sec.
0.005	8 min.	17:15	2 min. 51 sec.
		17:35	2 min. 50 sec.
0.005	32 min.	17:19	2 min. 41 sec.
		17:41	2 min. 43 sec.

a limit significantly short of 100% (table 4). This result suggests that a portion of the TA taken up by the cell is held much more firmly than the rest, the dissociation constant for this portion being so small that dissociation does not occur even after diluting 10^5 times.

The reversibility of the TA effect by protein obviously becomes a factor in any TA experiment involving hemolysis, since the first portion of the liberated hemoglobin acts

similary to gelatin and so diminishes the TA effect on the remaining cells. This behavior of hemoglobin can be demonstrated by comparing the degrees of retardation (R) at various points along the hemolysis curve. Unless some reversal of the TA effect occurs, R should be constant throughout the entire process. In figure 3 is shown the relationship of R to the end point selected for its calculation. It will be

TABLE 4

Effect of repeated washings in alkaline saline (pH 8.1) after a previous exposure of beef cells to 0.005% tannic acid at pH 6.8 for one hour

NUMBER OF WASHES	INITIAL TANNIC ACID CONCENTRATION %	TIME FOR 40% HEMOLYSIS IN M/6 NH_4Cl
5	0	5 min. 50 sec.
0	0.005	5 hours 3 min.
1	0.005	8 min. 20 sec.
2	0.005	8 min. 39 sec.
3	0.005	9 min. 40 sec.
5	0.005	8 min. 45 sec.

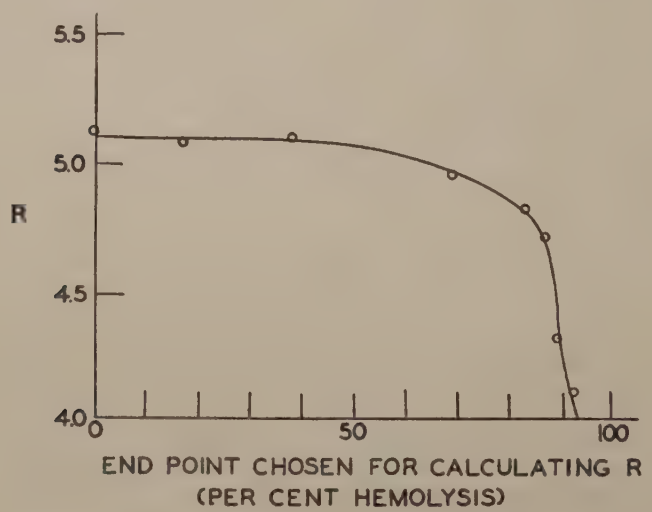

Fig. 3 Reduction in the effectiveness of tannic acid caused by the liberation of hemoglobin during hemolysis in M/6 NH_4Cl. Tannic acid concentration, 5×10^{-4}%. Values for R were extracted from complete swelling and hemolysis curves.

seen that the value of R falls off rapidly as the liberated hemoglobin exerts its reversing effect. In the quantitative work R was measured at 40% hemolysis, since at this point, with the dilute suspensions used, there is no significant reversal by liberated hemoglobin and the rate of change of optical density is rapid enough to furnish sharp end points.

Site of action

It is generally believed that the effects so far mentioned, and others, are a result of the action of TA on the cell surface. The various phenomena accompanying exposure of cells to TA which are mentioned in the Introduction implicity involve this portion of the cell. There are also several other lines of evidence to support the concept of a surface reaction.

For one thing, the instantaneous nature of the effect produced by a water-soluble molecule having the very high molecular weight of 1700 (Fischer and Freudenberg, '12; Merck and Co., '40) seems incompatible with any mechanism involving the interior of the cell. Then, too, the rapid reversibility of the effect produced by washing or by the addition of protein to the external medium seems to require the presence of TA on the accessible outer surface of the cell. Further evidence that the membrane actually does constitute an effective barrier to TA is provided by an experiment similar to one originally used by Jacobs in studying the effect of traces of copper. Hemoglobin is known to combine with TA to form a precipitate; therefore, if TA exerted its instantaneous effect by rapid penetration of the cell, then the hemoglobin in a sample of cells should inactivate TA whether it is imprisoned by membranes or liberated by hemolysis. However, it is found that the addition of an isotonic suspension of intact cells is far less effective in the inactivation of TA in M/6 NH_4Cl than is the addition of the same volume of isotonic hemolysate obtained from the same number of cells. The relative degree of inactivation can

be seen in the comparative hemolysis times when fresh batches of cells are added to the pre-treated solutions (table 5).

The effect of temperature on the amount of TA taken up by the cells in a given solution also points to a surface reaction. By a chemical method for the analysis of the residual TA (to be described in a later paper) it has been found that

TABLE 5

Effect of the addition of intact or hemolyzed cells upon the activity of a solution of tannic acid (1.6×10^{-4}%). The cell concentration was 9.4×10^{6} cells per milliliter. The number of intact or hemolyzed cells added to 10 ml of solution was 2.3×10^{7}

AGENT ADDED TO TANNIC ACID SOLUTION	TIME FOR 40% HEMOLYSIS		R
	Control	Tannic acid	
A.	5 min. 2 sec.	21 min. 58 sec.	4.38
B. Intact cells	5 min. 21 sec.	11 min. 20 sec.	2.12
C. Hemolyzed cells	4 min. 49 sec.	5 min. 27 sec.	1.11

the temperature data point toward a cell-TA complex whose dissociation increases with rising temperature. The amount of TA taken up by the cells from the same solution at different temperatures (pH 6.3) were as follows:

0.6°C. . . . 5.4×10^{-14} gm/cell
15.0°C. . . . 4.6×10^{-14} gm/cell
28.3°C. . . . 3.7×10^{-14} gm/cell
40.5°C. . . . 3.0×10^{-14} gm/cell

That TA can and does combine with the cell membrane is demonstrated by a rather disturbing complication. As has been mentioned previously, Stewart and Jacobs ('43) had shown that a substance capable of inactivating TA apparently escapes from the cell membrane when a washed cell suspension is allowed to stand in saline. This finding, demonstrated by restoration of the original sensitivity to TA after rewashing, was confirmed by the author. Furthermore, Kite ('14) observed that intact cells in saline gave off minute visible structures similar to those later described by Furchgott ('40) for ghosts.

The speed of action and magnitude of this complicating factor accompanying dilution may be illustrated by the following experiment. When part of a concentrated stock suspension of cells was centrifuged and a drop of the supernate added to a 10 ml portion of M/6 NH_4Cl containing $8 \times 10^{-5}\%$ TA, the biological activity of the solution in prolonging hemolysis was found to be reduced to that of a $6.7 \times 10^{-5}\%$ TA solution, the difference having been bound by agents in the drop of supernate. On the other hand, if instead, a drop of the stock suspension were first diluted in 10 ml of M/6 NH_4Cl and this portion centrifuged within 45 seconds, during which period no hemolysis occurs, it was found that upon adding to the cell-free supernatant liquid sufficient TA to produce a concentration of $8 \times 10^{-5}\%$, the biological activity was only $4 \times 10^{-5}\%$. That this greater amount of released substance in the second case was due to dilution of the suspension and not to centrifugation is indicated by the fact that varying the centrifugal force by a factor of 23 made no difference in the amount of inactivation. Since numerous biological agents may combine with either protein or phospholipid, which are two of the possible constituents of the released material (Furchgott, '40), the importance of considering this phenomenon in indirect approaches to similar problems is considerable.

In the light of these various lines of evidence, it seems difficult to accept Bohlmann's hypothesis ('44) of TA action by penetration of the cell. With the preponderance of evidence in favor of a reaction of TA with the cell surface as the origin of the effects so far described, it is interesting to examine further some of the data previously presented above. It will be recalled that for a suspension containing 5500 cells per cubic millimeter, at a TA concentration of $2.5 \times 10^{-7}\%$, a reproducible value of 1.17 was obtained for R. Taking the molecular weight of TA as 1700, the maximum area covered by a single TA molecule as 650 $Å^2$ (author's calculation) and the surface area of the human erythrocyte as 1.6×10^{10} $Å^2$ (Ponder, '48), it can be calculated that at

most only 0.66% of the cell surface could have been covered by TA under the above conditions. If the covering of less than 1% of the surface results in a 17% increase in hemolysis time, it is rather likely that only a small fraction of the normal cell surface is permeable to ions. Furthermore, the fact that the movement of most non-electrolytes, unlike that of ions, is unaffected by the concentrations of TA in question may point to separate routes of entrance for each.

Effects on osmotic resistance

Bohlmann ('44) reported confirmation of the original observation of Handovsky and Masaki ('23) that TA decreases the osmotic resistance of the erythrocyte. Since it is important for purposes of comparison to maintain the hemolytic volume constant, experiments were conducted to determine the effect of TA upon osmotic resistance over the concentration range used in the present study.

After a 10 minute exposure of a suspension to hypotonic saline containing TA at a controlled temperature and pH, the sample was centrifuged and the concentration of hemoglobin in the supernate determined colorimetrically. Results for a suspension containing 4400 cells per cubic millimeter, a concentration typical of those used here, are shown in figure 4. It is seen that the decrease in osmotic resistance does not occur below a certain minimum concentration of TA and that it soon reaches a maximum value which remains constant. The fact that increase in fragility does not start until the concentration of TA reaches $5 \times 10^{-6}\%$ leads to an interesting situation. If the effect of TA on ionic movement is schematically represented as a factor which starts at zero TA concentration to prolong hemolysis, and the effect on osmotic resistance as a factor which starts at $5 \times 10^{-6}\%$ TA to accelerate hemolysis, algebraic addition of the two curves should give a wave-like curve as illustrated in figure 5 A.

A curve of this sort may in fact be obtained experimentally as is shown in figure 5 B. It may be noted that the first zone of retarded hemolysis is the range in which the minimum effective concentrations were observed by the use of the alternate method mentioned above on page 534. The balance between the two factors in this zone is very delicate, slightly

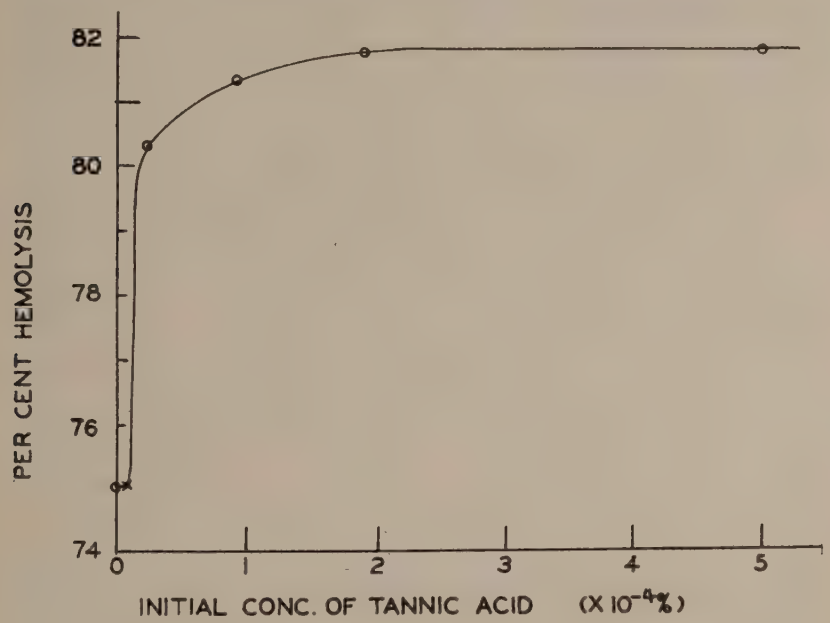

Fig. 4 Variation in osmotic resistance with concentration of tannic acid. 0.48% NaCl, pH 6.3, 23°C., 4400 cells per cubic millimeter. The lower range was obtained from experiments involving smaller increments of tannic acid.

too many cells or too much protein decreasing the retardation to an unrecognizable level, and slightly too much TA carrying the sample into the zone of accelerated hemolysis. It is believed that the alternate method, by increasing osmotic resistance and by using for observation the more resistant portion of the cell population (end point at 80% hemolysis), places the system in the range where retardation of hemolysis may be observed.

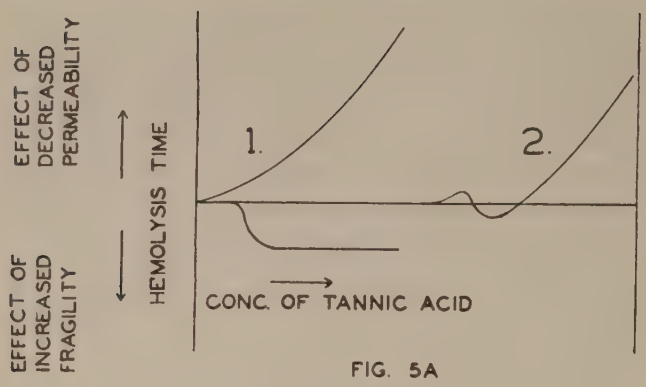

FIG. 5A

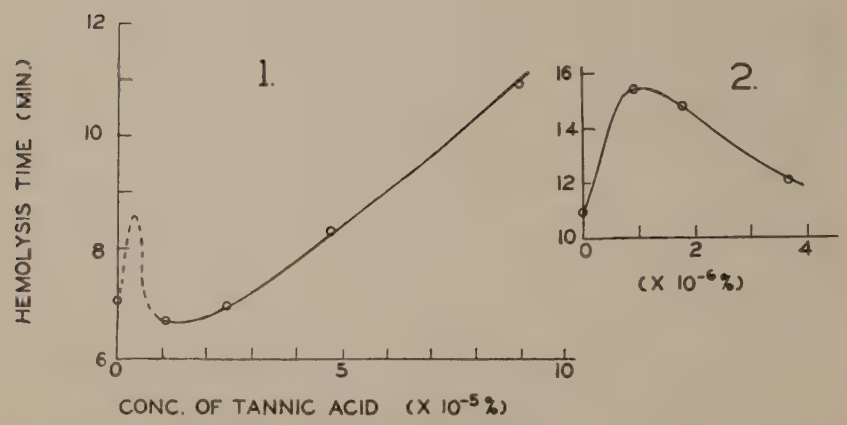

FIG. 5B

Fig. 5 Interaction between the increase in fragility and the decrease in permeability produced by tannic acid and its effect on the time of hemolysis in M/6 NH_4Cl.

A Theoretical curves: (1) separate effects plotted schematically; (2) algebraic summation of the effects in (1).

B Experimental results: (1) curve obtained by the usual method; (2) curve obtained at the extremely low region of the concentration range, using the alternate method.

Effect on hemolysis by non-electrolytes

Although Jacobs, Stewart and Butler ('43) reported hemolysis in numerous non-electrolyte solutions to be virtually

unaffected by low concentrations of TA, there are two cases worthy of note in which this is not so. Jacobs and Stewart (unpublished) found that the permeability of human cells to glycerol at acid pH was greatly retarded by TA. These findings were confirmed by the author (table 6), the permeability of beef cells under similar conditions being unaffected. It was further discovered that pigeon blood cells which are also very permeable to glycerol, behave like human cells in having their permeability to glycerol greatly diminished by

TABLE 6

Effect of tannic acid on the permeability of beef, human and pigeon erythrocytes to glycerol. Solution contained 0.3 M glycerol, 0.09% NaCl and a phosphate-phthalate buffer. The cell suspensions were at the same pH as that of the glycerol solution used for each

SPECIES	pH	TIME FOR 40% HEMOLYSIS		
		Control	0.005% tannic acid	0.0005% tannic acid
Beef	7.2	53 min.	55 min.	
	6.4	19 min. 21 sec.	19 min. 55 sec.	
Human	7.9	43 sec.	42 sec.	
	7.0	45 sec.	6 min. 24 sec.	
	6.1	15 min. 36 sec.	27 min. 45 sec.	
Pigeon	7.3	3 sec.		3 sec.
	6.8	4 sec.		4 sec.
	6.2	7 min. 50 sec.		20 min. 9 sec.

TA at acid pH (table 6). On the basis of a similar specific inhibition of glycerol hemolysis by copper (Jacobs and Corson, '34) and by certain inhibitors of enzymes (LeFevre, '47), it appears that in this case a special mechanism unrelated to the general permeability effect may be involved Jacobs, '50).

Tannic acid may, oddly enough, produce an acceleration of hemolysis in various non-electrolyte solutions which superficially resembles an increase in permeability. This acceleration can be shown in fact to be due to a retardation of the ionic shift which would normally occur in pure non-electro-

lyte solutions and which would cause the cells to shrink (Jacobs, Parpart and Corson, '37), thereby opposing hemolysis. The accelerating affect may readily be prevented by the addition of a small amount of saline (table 7) which removes the shrinkage effect of the non-electrolyte solution.

TABLE 7

Effect of tannic acid on hemolysis time of beef cells in glycerol in the presence and absence of electrolyte, pH 6.4

HEMOLYZING MEDIUM	TIME FOR 40% HEMOLYSIS	
	Control	0.005% tannic acid
0.3 M glycerol	27 min. 34 sec.	19 min. 9 sec.
0.3 M glycerol containing 0.09% NaCl	19 min. 33 sec.	19 min. 42 sec.

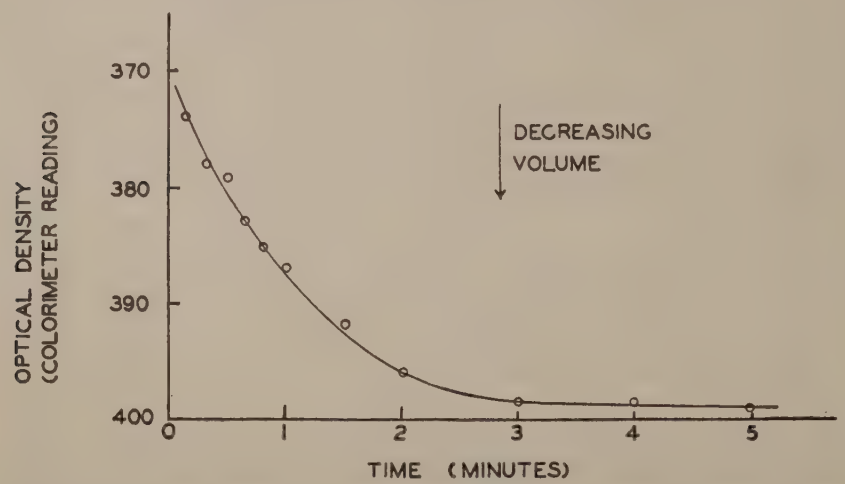

Fig. 6 Shrinkage curve for beef erythrocytes in M/9 Na_2SO_4 obtained by the tannic acid method.

Use of TA as a research tool

The extreme retardation of ionic movement produced by TA may profitably be applied as a research tool. For example, Jacobs, Stewart and Butler ('43) demonstrated the initial penetration of NH_3 into the cells when NH_4OH is added to the medium, by using TA to eliminate the masking effect

of the subsequent ion exchange. The author has been able to follow the course of shrinkage of erythrocytes in a solution of Na_2SO_4 (as a result of the exchange of pairs of Cl^- ions for single $SO_4^=$ ions) by "freezing" the process at intervals with TA so that photoelectric observation of volume changes could be made at leisure. The method consists of starting the process by mixing washed cells with a large volume of M/9 Na_2SO_4 (pH 7) and of drawing off 10 ml portions at desired intervals. These portions are squirted into tubes, each containing one drop of 1% TA. Readings are made on the colorimeter within 20 minutes after transfer and results plotted as in figure 6.

SUMMARY

1. Special precautions, which are described, must be taken to obtain accurate results in experiments involving the use of very dilute solutions of tannic acid.

2. Tannic acid in very low concentrations is capable of decreasing the rate of hemolysis in solutions of NH_4Cl by a factor of 200.

3. Concentrations of tannic acid as low as 1.5×10^{-9} M may appreciably retard hemolysis in NH_4Cl.

4. There is no apparent effect by tannic acid at similar concentrations on the permeability of erythrocytes to most non-electrolytes, glycerol being a notable exception in some species.

5. The effect of tannic acid upon permeability to ions may be completely reversed by the addition of protein but is only incompletely reversed by repeated washing of the cells.

6. The effect on permeability is accompanied by a decrease in osmotic resistance.

7. The evidence strongly indicates that the site of the action of tannic acid is the surface of the cell.

8. The results obtained suggest that only a relatively small fraction of the cell surface is permeable to anions.

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